On the Evolution of Sperm Morphology and Sperm-Related Genes in a Howler Monkey Hybrid System

by

Molly Amber Hirst

A dissertation submitted in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy
(Ecology and Evolutionary Biology)
in the University of Michigan
2023

Doctoral Committee:

Research Associate Professor Liliana Cortés-Ortiz, Co-Chair
Professor Patricia J. Wittkopp, Co-Chair
Associate Professor Jacob Mueller
Associate Research Scientist Cody Thompson

Molly Amber Hirst

mhirst@umich.edu

ORCID iD: 0000-0003-0321-7048

© Molly Amber Hirst 2023

Dedication

For my nephew Karson, the light of my life.

For my dad and mom, without whom I could not have gotten this far.

For my sister, brother, Korri, Xorla, Yao, Lisa, Amberly, Stephanie, and Roy for their never-ending love and support.

For Mason Charles Hirst, who was always so proud of me;

and for Virginia Alice Hirst, who still is.

For everyone else who supported me in various ways throughout the years.

For all the first-generation, underrepresented students. I believe in you.

Acknowledgements

I could not have completed this dissertation without the following people and resources. First, a huge thank you to my main doctoral advisor, Dr. Liliana Cortés Ortiz, who taught me how to be a better scientist and who has been exceedingly supportive both professionally and personally. Thank you to my secondary advisor Trisha Wittkopp, who always made time to read my manuscript drafts, talk through statistics, and be an excellent role model. Thank you to my committee member Jake Mueller, who always asked pertinent questions and provided some of the best methods advice and ideas. Thank you to my committee member Cody Thompson, who helped me foster a grand appreciation for biological museum research and outreach and was always willing to discuss everything from evolutionary theory and ethics of specimen usage to permits. Thank you to all of my collaborators and co-authors, whose knowledge, guidance, and friendship have been some of my greatest treasures: Alba Z. Rodas-Martínez, Krista Milich, Mary A. Kelaita, Susanna J. K. Gutierrez, Marcella D. Baiz, Alexandre Inforzato, Amy Goldberg, Domingo Canales-Espinosa, and Patricia Domingues de Freitas. I have been immensely fortunate and thankful to have known and worked alongside so many of the Cortés-Ortiz and Wittkopp Lab members past and present. A huge thank you to our Mexican field collaborators, without whom this work would have been impossible.

This research was supported by the University of Michigan's (UM) Department of Ecology and Evolutionary Biology, the Rackham Graduate School, the Dr. Nancy Williams Walls Award for Field Research, the UM Department of Anthropology, the American Society of Mammalogists Grants-in-aid, and the International Primatological Society. I was also supported

by the Rackham Merit Fellowship program and the NIH training grant “Michigan predoctoral Training in Genetics (T32GM007544)”.

Table of Contents

Dedication ii

Acknowledgements iii

Table of Contents v

List of Tables viii

List of Figures ix

Abstract x

Chapter 1 : Introduction 1

1.1 Sexual selection acts on sperm morphology 1

1.2 Hybrid zones and speciation 5

1.3 Hybrid zones and sperm morphology 8

1.3.1 The Mexican howler monkey hybrid zone 10

1.4 Introduction to this dissertation 11

Chapter 2 : Differences in Sperm Morphology Between *Alouatta palliata* and *A. pigra* are Consistent with the Intensity of Sperm Competition in Each Species 14

2.1 Abstract 14

2.2 Introduction 15

2.3 Materials and Methods 19

2.3.1 Ethical Note and Data Availability 19

2.3.2 Sampling 19

2.3.3 Semen collection and sperm slide preparation 20

2.3.4 Sperm measurements 22

2.3.5 Reliability of sperm morphology measurements 22

2.3.6 Statistical analyses to determine differences in sperm traits between species 23
2.3.7 Evaluation of the proportion of normal versus abnormal sperm 24
2.4 Results 25
2.4.1 Sperm abnormalities 25
2.5 Discussion 26
2.5.1 Why are sperm midpiece and head longer in A. palliata? 26
2.5.2 Is sperm length constrained in Alouatta? 29
2.6 Conclusion 31
Chapter 3 : Directional Bias in the Occurrence of Sperm Morphological and Testicular Abnormalities in Highly Backcrossed Howler Monkey Hybrids 44
3.1 Abstract 44
3.2 Introduction 45
3.3 Materials and Methods 50
3.3.1 Ethical note and data availability 50
3.3.2 Sample collection 50
3.3.3 Hybrid index 51
3.3.4 Sperm morphology data collection and reliability 52
3.3.5 Statistical analysis of sperm morphology 53
3.3.6 Abnormal sperm morphology 54
3.3.7 Abnormal testicular morphology 55
3.4 Results 55
3.4.1 Sperm morphology 55
3.4.2 Abnormal sperm morphology 56
3.4.3 Abnormal testicular size and morphology 57
3.5 Discussion 58
3.6 Conclusion 63

Chapter 4 : Purifying Selection and Complex Patterns of Introgression of Putative Sperm-Related Genes in a Howler Monkey Hybrid Zone 72

4.1 Abstract 72

4.2 Introduction 74

4.3 Materials and Methods 79

4.3.1 Ethical note 79

4.3.2 Sampling and choice of A. pigra individual for de novo whole genome sequencing 80

4.3.3 DNA extractions and whole genome sequencing 80

4.3.4 Alouatta pigra genome assembly 81

4.3.5 Identification of putative sperm-related genes in howler monkeys 82

4.3.6 Tests of selection 83

4.3.7 Patterns of introgression and links with archaic hominin hybridization 84

4.4 Results 85

4.4.1 de novo genome assembly 85

4.4.2 Identification of putative Alouatta sperm-related genes 86

4.4.3 Analysis of selection on putative Alouatta sperm-related genes 86

4.4.4 Patterns of introgression in the hybrid zone and associations with archaic ancestry in humans 87

4.5 Discussion 89

4.5.1 Most putative Alouatta sperm-related genes show signatures of purifying selection 91

4.5.2 Introgression patterns of putative sperm-related genes are complex 92

4.5.3 Links between gene regions with increased or reduced archaic hominin hybridization and putative sperm-related gene introgression in howler monkeys 94

4.6 Conclusion 97

Chapter 5 : Conclusions 104

Bibliography 108

List of Tables

Table 2-1: Summary of relevant *Alouatta palliata* and *A. pigra* sexually selected traits and predicted sperm morphology traits. 33

Table 2-2: Model estimates of fixed and random effects using raw data. 34

Table 2-3: Mean sperm measurements for each individual. 35

Table 2-4: Repeated measures correlation matrix between sperm measurements within individuals. 36

Table 2-5: Average sperm total lengths, midpiece lengths, and head lengths of diverse primates from published sources. 37

Table 3-1: Testicular details for unadmixed and admixed male subjects. 64

Table 3-2: Sperm morphology measurements and details for admixed individuals. 65

Table 3-3: Analyzing variance in sperm morphology across group categories and hybrid index. 66

Table 3-4: Classification and statistics of sperm abnormalities per group category. 67

Table 3-5: Analysis of sperm abnormality across group categories. 68

Table 4-1: Bioinformatics pipeline for building the de novo *Alouatta pigra* genome assembly. 99

Table 4-2: Reference genome assembly statistics comparison between *Alouatta pigra* and *A. palliata*. 101

Table 4-3: Twenty-one genes found in both *Alouatta* reference genome assemblies and analyzed for selection and SNP introgression. 102

List of Figures

Figure 2-1: Sampling localities of individuals analyzed in this study and distribution ranges of each species. 41

Figure 2-2: Diagram of measurements taken on sperm cells. 42

Figure 2-3: Violin plots of sperm head length and midpiece length between species. 43

Figure 3-1: Ranges and sampling sites of *Alouatta palliata*, *A. pigra*, and the hybrid zone in Southern Mexico. 69

Figure 3-2: Sperm morphological abnormalities in howler monkeys. 70

Figure 3-3: Effect of hybridization on sperm head and midpiece lengths. 71

Abstract

Sperm are among the most morphologically diverse animal cell. This diversity is surprising because mutations in genes responsible for sperm morphology can impair reproductive success, thus there should be strong constraints to variation. Sperm morphological diversity is mainly driven by sexual selection when novel adaptive traits enable a male's sperm to outcompete other male's sperm during fertilization. Sperm diversity may also increase due to selection against producing hybrid offspring between closely related species, as sperm morphological differences in sympatry increase, reinforcing reproductive barriers. Despite the importance of post-mating reproductive barriers for speciation, identifying these barriers and the types of selection acting on specific phenotypes is complicated. My dissertation addresses how differences in male reproductive traits between two howler monkey species, including sperm morphology, testicular abnormalities, and genetics, relate to reproductive isolation and adaptation.

The *Alouatta palliata* x *A. pigra* hybrid zone in southern Mexico is an excellent system to address my research questions as these species differ in mating system and other relevant life history characteristics as reviewed in chapter 1. In chapter 2, I characterize sperm morphological differences between these species. Given that *A. palliata* is polygynandrous and has twice the testicular volume relative to body size as *A. pigra*, I hypothesized that *A. palliata* sperm exhibit morphology indicative of more intense sperm competition than those in *A. pigra*. Indeed, I found longer sperm heads and midpieces in *A. palliata*; traits associated with fast sperm in other taxa.

Provided that longer sperm heads and midpieces are likely advantageous, in chapter 3 I hypothesized that normal sperm in hybrids have long heads and midpieces. However, I found

that *A. palliata*-like hybrids (i.e., highly backcrossed into *A. palliata*) have sperm with short midpieces, and higher proportions of abnormal sperm and testicular morphology. These results suggest stronger negative effects of hybridization on *A. palliata*-like fitness compared to *A. pigra*-like hybrids.

In chapter 4, I explored patterns of selection and introgression of *Alouatta* genes that are homologs to a set of genes essential for human sperm function. I generated the first *de novo* genome assembly for *A. pigra* and used the published *A. palliata* genome assembly to identify homologs to these "sperm-related genes" and compare their sequences. I found that 17/21 genes associated with sperm development and function are under purifying selection between these species. I leveraged genome-wide SNP data previously published for this hybrid system and found nine SNPs near (and thus likely associated with) these putative sperm-related genes. Most of these SNPs show excess *A. palliata* ancestry in the hybrid zone, and approximately equal instances of reduced and increased introgression. Two of the SNPs with reduced introgression also show elevated expression in human testes. It is possible that some genes are involved in other genomic pathways under different selective pressures, resulting in the mixed introgression patterns. Lastly, I looked for these genes in known regions of elevated/depleted archaic ancestry in humans, indicating candidates for adaptive or barrier loci, respectively, in primates.

Chapter 1 : Introduction

Sperm are among the most morphologically diverse animal cell across taxa and can exhibit stark differences between closely related species. Such diversity arises from a complex interplay of factors, which are not mutually exclusive, including sexual selection via sperm competition, cryptic female choice, and sexual conflict, in addition to drift. My dissertation focuses on sperm morphology and sperm genetics in a howler monkey hybrid zone. By analyzing the variation in sperm morphological traits, I aim to uncover how these differences may drive adaptation or contribute to reproductive barriers in this hybrid system. This introductory chapter sets the stage for my research by offering a theoretical framework to sexual selection, sperm biology, hybrid zones, and the specifics of my study system.

1.1 Sexual selection acts on sperm morphology

Sexual selection acts on sexual traits, such as sperm morphology and testicular size and shape, and favors those traits that result in increased reproductive fitness. Sexual selection can occur before copulation, resulting in traits easily observable such as male peacock tail feathers (e.g., Møller & Höglund, 1991; Smith, 1991; Loyau et al., 2007), and after. Post-copulatory sexual selection occurs via sperm competition, cryptic female choice, and sexual conflict, or a combination of them (e.g., Eberhard, 2009; Firman et al., 2017; Firman, 2020; Hosken, Archer, Mank, 2019). Sperm competition occurs when sperm compete to reach and fertilize an egg (Parker 1970; Parker & Partridge, 1998). The mechanisms of sperm competition vary widely across species and can include, for example, increased sperm production (Parker, 1993; Lüpold & Fitzpatrick, 2015), sperm morphological adaptations (Lüpold & Pitnick, 2018; Lüpold et al.,

2020), and ejaculate adjustment based on the level of expected competition (Magris, 2021; Pound & Gage, 2004). Although more difficult to measure (but see Poignet et al., 2022 for novel methods), cryptic female choice is female-biased mediation of sperm choice based on compatibility, genetic quality, or diversity (e.g., Firman et al., 2017; Eberhard, 1996). This can occur through sperm storage where certain sperm from different males can be selected to use for fertilization at a given time, and sperm manipulation where females can manipulate the longevity or motility of sperm, influencing which sperm are likely to succeed in fertilization (reviewed in Firman et al. 2017; Firman, 2020). In mammals, complex, elongated oviducts in females are associated with larger relative testes size and sperm midpieces in males, suggesting cryptic female choice has influenced the coevolution of these male and female reproductive traits (Anderson, Dixson, Dixson, 2006). However, this pattern is poorly understood in primates due to the difficulties in obtaining these types of data from non-human primates. Sexual conflict arises when the reproductive fitness optima of males and females diverge, particularly over the mode and frequency of reproduction, leading to an evolutionary arms race between the sexes (e.g., Hosken, Archer, Mank, 2019; Parker & Partridge, 1998; Rowe et al., 2020; Stumpf et al., 2011; Svensson, 2018). In this case, the discordance occurs when a beneficial allele in one sex spreads, regardless of its cost to the opposite sex, so long as the benefit for the one sex outweighs the cost for the other (Parker, 1979). In *Drosophila melanogaster*, for example, sex peptide in the seminal fluid is toxic to females, reducing their lifespan and decreasing the ability of the female to remate and increasing the likelihood of paternity of the resulting offspring (Chen et al., 1988; reviewed in Hopkins & Perry, 2022). Evidence for sexual conflict in primates is documented mostly for pre-copulatory and copulatory traits such as sexual swellings that extend the length that sperm need to swim, and penile spines in galagos that can damage the female reproductive tract

(reviewed in Dixson, 2018). Some evidence for post-copulatory mechanisms of sexual conflict, such as embryo reabsorption after exposure to a new male's sperm, and fluids secreted during female orgasm that affect the survival of sperm, have been occasionally documented in primates (reviewed in Stumpf et al., 2011).

The intensity of post-copulatory sexual selection, which acts on both the male and female reproductive tracts and fluids, is associated with mating system in primates. After copulation occurs in internal fertilizers, millions of sperm with unique recombined haplotypes compete to reach and fertilize eggs. However, because each sperm, regardless of the haplotype it carries, comes from the same male, the stakes for selection are low if the female only mates with one male, because his paternity is nearly guaranteed. In cases where females mate with multiple males at a given time, such as in species with polyandrous (in which one female mates with multiple males) and polygynandrous (in which multiple females mate with multiple males) mating systems, sperm from multiple males compete for fertilization inside the female's reproductive tract. This sperm competition can be intense, leading to selection of specific traits that help sperm outcompete other sperm (e.g., Parker, 1970; Lüpold & Pitnick, 2018). This can be due by increasing the number of sperm or through morphological variation in different parts of the sperm.

Intense sperm competition results in an increase in sperm-producing tissue, which manifests into large testicles (Harcourt et al., 1981; Møller, 1988; Møller, 1989; Harcourt et al., 1995; Lüpold et al., 2020). There is evidence for a correlation between testes volume and sperm production (e.g., Takihara et al., 1987; Lüpold, Simmons, Gureter, 2019; Møller, 1988). Therefore, differences in testicular size can be used to infer the intensity of sperm competition in a species. For example, the ratio of testes to body size, or gonadosomatic ratio, indicates the

amount of sperm that a male can theoretically produce (e.g., Sharpe et al., 2003; Papaioannou et al., 2009; Griswold, 1998), and has been used to infer sperm competition in different taxa, especially in primates where mating systems are diverse and complex (Harcourt et al., 1981; Harcourt et al., 1995; Møller, 1988).

Sperm morphological traits can also provide information about the intensity of sexual selection in species. Indeed, sperm cells are some of the most morphologically diverse animal cells, with morphological traits differing between species and even between populations (e.g., Pitnick, Hosken, Birkhead, 2009; Lüpold & Pitnick, 2018). Although they are vastly morphologically diverse, the only function of sperm is to locate and fertilize a female's gamete. As such, it may seem counterintuitive that such widespread morphological diversity exists among organisms, given their vital function in reproduction. Yet, evidence built across the tree of life suggests several factors are responsible for this diversity (e.g., Parker, 1970; Parker & Partridge, 1998; Pitnick, Hosken, Birkhead, 2009; Lüpold & Pitnick, 2018). One important factor is the mode of fertilization. Some taxa are internal fertilizers in which sperm are directly transferred to the female reproductive tract, other taxa are external fertilizers in which both male and female gametes are dispersed in an aquatic medium, and yet other taxa are spermcasters in which sperm are released in an aquatic environment and females take in the sperm into their reproductive tracts from the aquatic environment. Kahrl, Snook, and Fitzpatrick (2021) compared sperm length diversification across >3,000 species from 21 phyla that represented different fertilization modes. They found that sperm are shorter in external fertilizers and spermcasters, which rely on aquatic environments to disperse their gametes, than in internal fertilizers, which have longer sperm. Additionally, in both internal fertilizers and spermcasters, sperm length evolves faster and undergoes more adaptive shifts, likely due to the interactions

with the female reproductive tract (Kahrl, Snook, Fitzpatrick, 2021). In primates, sperm competition in particular is expected to play a large role in sperm morphology (e.g., Martinez & Garcia, 2020), especially given the vast diversity of mating systems among and between primates (e.g., Harcourt, Purvis, Liles, 1995). However, how different intensities of sperm competition affects sperm morphology in wild primates is unknown.

1.2 Hybrid zones and speciation

Hybrid zones are geographical areas where species meet, mate, and produce at least some viable offspring (Harrison 1993). In specific cases referred to as tension zones, there is a balance between dispersal of unadmixed individuals of parental species into the hybrid zone and selection against hybrids (Barton & Hewitt, 1985). Tension zones are thus natural laboratories in which admixed genotypes that have never been tested in a natural environment can be studied to understand the process of speciation. Through instances of hybridization and the study of hybrid zones from various taxa, it is well-established that two general rules of speciation apply to most systems. First, Haldane's Rule posits that hybrids of the heterogametic sex (e.g., XY or ZW) are likely to be sterile or inviable (e.g., Haldane, 1922; Cowell, 2023). This could be due to several reasons, including but not limited to genetic dominance effects, faster male gene evolution, and negative epistatic interactions (reviewed in Cowell, 2023). For example, negative effects of incompatible genes from different species might be recessive and hence masked in the homogametic sex but exposed in the heterogametic sex (reviewed in Cowell, 2023). The faster-male hypothesis suggests that male reproductive genes evolve faster than those of females, and as a result, differences in male reproductive genes between species are likely more pronounced, leading to reduced hybrid male fertility or viability (reviewed in Cowell, 2023). Lastly, differential gene interactions can occur because the Y (or W) chromosome does not recombine

over most of its length, which can lead to the accumulation of mutations or degenerative alleles resulting in negative interactions with the X chromosome of the other species (reviewed in Cowell, 2023). In the homogametic sex, the presence of alleles from a second X chromosome can oftentimes rescue any negative effects of the first X chromosome (reviewed in Cowell, 2023). Second, the X chromosome is known to have a disproportionately large effect of hybrid sterility and inviability compared to autosomes (e.g., Presgraves, 2018; Coyne & Orr, 2004). For example, heterogametic males will be exposed to any recessive alleles on their hemizygous X chromosome, whereas those recessive effects can be masked in the homogametic sex when there is a functional allele on the other X chromosome (reviewed in Cowell, 2023). Additionally, males with only one X chromosome often exhibit upregulated gene expression to compensate for the missing second X chromosome, and mismatches in regulatory mechanisms between hybridizing species can lead to maladaptive gene expression in hybrids (reviewed in Cowell, 2023). Lastly, negative interactions between the Y chromosome from one species and the X chromosome in another species can result in hybrid dysfunction, especially given the prevalence of male-specific genes on the Y chromosome (e.g., Kopania et al., 2022). The large effect of the X chromosome further mediates incipient speciation between hybridizing species. Together, these two rules of speciation contribute to hybrid sterility and inviability. Thus, it is possible to identify traits that contribute to reproductive isolation between two species by studying hybrid zones.

Hybrid zones have been described as windows into the evolutionary process (Harrison, 1990) such that the factors contributing to adaptation and reproductive isolation can be observed. Specifically, hybridization and recombination shuffle divergent genomes resulting in hybrid genomes with novel allelic combinations subject to selection (e.g., Barton & Hewitt, 1985;

Harrison, 1990; 1993; Harrison & Larson, 2014; Larson et al., 2014; Taylor & Larson, 2019). In my dissertation, I study a primate hybrid zone, and the subsequent discussion of hybrid zones is based on animal hybrid zones, but an extensive review of plant hybrid zones can be found in Abbott (2017). Most hybrid zones are not comprised of F1 individuals (i.e., a hybrid produced from non-admixed parents from both species) but consist of hybrids backcrossed with one species or the other, or that are the product or mating between hybrids. These individuals are referred as multigenerational backcrossed individuals (e.g., Harrison, 1993). When alleles or combinations of alleles work well in the hybrid genome and provide an advantage to individuals who have them, they introgress or pass, from one species to the other at a faster rate than the rest of the genome (e.g., Barton & Hewitt, 1985; Buerkle, 2005). In contrast, genomic regions that contribute to hybrid sterility, inviability, or other maladaptive phenotypes are considered to be barriers and exhibit either no or reduced rates of introgression when compared to the rest of the genome (e.g., Barton & Hewitt, 1985; Buerkle, 2005). As such, estimating introgression of genomic regions using genomic cline analysis is an important step in understanding how interspecific variants, for example, introgress in the hybrid zone compared to the expected pattern of genome-wide neutrality (e.g., Szyumra & Barton, 1986; Gompert & Buerkle, 2012). Specifically, genomic cline analysis relies on the extent of genetic admixture of individuals in the hybrid zone, which can be measured as a hybrid index (e.g., Buerkle, 2005). The genetic hybrid index is a scale from 0 to 1 where 0 indicates unadmixed individuals from one species, and 1 indicates unadmixed individuals from the other species. Individuals with a genetic hybrid index between 0 and 1 have some proportion of their genome derived from one parental species and the rest from the other (e.g., Buerkle, 2005; Gompert & Buerkle, 2012). Analyzing how each allele introgresses through the hybrid zone via clinal analysis provides insight into the rate and

direction of introgression (e.g., Barton & Hewitt, 1985; Gompert & Buerkle, 2012). For example, alleles with a steep cline likely do not pass through the hybrid zone and are considered to be barrier loci (e.g., Gompert & Buerkle, 2012), whereas alleles from one of the parental species that are overrepresented in hybrid individuals across the hybrid index likely represent adaptive alleles. Therefore, it is possible to follow traits across hybrid zones to determine how certain traits differ in either parental species and how they manifest in hybrids to develop a sense of whether or not those traits may be adaptive or act as barriers to reproduction in the hybrid zone (e.g., Barton & Hewitt, 1985; Gay et al., 2008).

1.3 Hybrid zones and sperm morphology

The effects of hybridization within hybrid zones on animal sperm morphology have been studied in some taxa (e.g., *Spea bombifrons x S. multiplicata:* Wünch & Pfennig, 2013; *Ammospiza caudacuta x. A. nelsoni:* Cramer et al., 2021; *Luscinia luscinia x. L. megarhynchos:* Albrecht et al., 2019; *Ficedula hypoleuca x F. albicollis:* Ålund et al., 2013; *Mus musculus musculus x M. musculus domesticus:* Albrechtová et al., 2012) and is in general quite variable. For example, selection for divergent sperm traits, such as morphology or motility, can be exacerbated when heterospecific matings occur. This is interpreted as a case of reinforcement to prevent the production of hybrid offspring, which are often unfit or have lower fitness (e.g., Howard et al. 1998; Geyer & Palumbi, 2007; Cramer et al., 2016). Selection against costly hybrid offspring is common, and one of the first phenotypes affected in hybrid males is fertility, often via disrupted sperm development or function (e.g., in mules: Steiner & Ryder, 2013; Chandley et al., 1974; in mice: Schwahn et al., 2018; Kopania et al., 2022; White et al., 2012; in toads: Wünsch & Pfennig, 2013).

Comprehensive sperm morphological traits have mostly been assessed in avian hybrid zones and in the house mouse hybrid zone. For example, sperm number, head abnormalities, motility, and velocity have been assessed in a natural house mouse hybrid zone in Europe between *Mus musculus musculus* x *M. musculus domesticus* (e.g., Turner & Harr, 2012; Bímová et al., 2020; Campbell & Nachman, 2014; Albrechtová et al., 2012; 2014). Importantly, Albrechtová et al., 2014 compared sperm morphology between wild-caught individuals and wild-derived strains from the house mouse hybrid zone and found that *M. musculus musculus* were characterized by larger sperm heads and midpieces, but *M. musculus domesticus* averages more sperm per ejaculate. Although both subspecies are considered to be polygynandrous (e.g., Thornhauser et al., 2013 and references therein), the results from this study suggest that there may be a trade-off in production of larger sperm in *M. musculus musculus* and the number of sperm they can per ejaculate (Albrechtová et al., 2014). In the avian *Luscinia megarhybchos* x *L. Luscinia* hybrid zone in Europe, sperm morphological divergence in sympatry, but not in allopatry, suggests a possible role of reinforcement in the reproductive isolation between these species (Albrecht et al., 2019). In the *Ficedula hypoleuca* x *F. albicollis* hybrid zone in Sweden, most males do not produce sperm but those that do produce malformed sperm and suffer reduced fertility (Ålund et al., 2013). Conversely, in North America, sperm length divergence between *Ammospiza caudacuta* and *A. nelsoni* does not appear to affect hybrid sperm in that they are morphologically and functionally normal (Cramer et al., 2021). Many factors, including strength of sexual selection and phylogenetic proximity, likely play a role in why and how hybridization affects sperm in different systems. Thus, the characterization and analysis of sperm across hybrid zone transects is important for detangling the mechanisms affecting sperm in hybrid zones across various taxa.

1.3.1 The Mexican howler monkey hybrid zone

A well-characterized hybrid zone system in primates is the *Alouatta palliata* x *A. pigra* howler monkey hybrid zone in Tabasco, Mexico (Cortés-Ortiz et al., 2007; 2019). This hybrid system is the focus of my dissertation for several key reasons. These species diverged approximately 3 million years ago (Cortés-Ortiz et al., 2003), thus providing ample opportunity for speciation to occur. Indeed, these species differ vastly in several traits important for reproduction, including mating systems (Wang & Milton, 2003; Ryan et al., 2018; Jones, 1985; Corewyn, 2015; Jones & Cortés-Ortiz, 1998; Bolin, 1981; Van Belle & Estrada, 2008; Van Belle et al., 2009a; 2009b), social systems and behavior (Treves, 2001; Van Belle et al., 2012; Ho et al., 2014; Baiz & Cortés-Ortiz, 2015), vocalizations (Kitchen et al., 2018), chromosome numbers (Steinberg et al., 2014), gonadosomatic index (Kelaita et al., 2011), and sperm morphology (Hirst et al., 2023; Chapter 2 of this dissertation), among other traits. Specifically, *A. palliata* social groups tend to consist of multiple males, multiple females, and their offspring. They are characterized as having a polygynandrous mating system, where females have opportunities to mate with multiple males in a given estrous period (Wang & Milton, 2003; Ryan et al., 2018; Jones, 1985; Corewyn, 2015; Jones & Cortés-Ortiz, 1998). In contrast, *A. pigra* are mostly polygynous, with social groups consisting of a central male, several females, and their offspring (Bolin, 1981; Van Belle & Estrada, 2008; Van Belle et al., 2009a; 2009b). As such, selection for adaptive sperm traits is hypothesized to be more intense in *A. palliata* than in *A. pigra*, and the former species has a high gonadosomatic index compared to the later (Kelaita et al., 2011). These two species meet in a narrow (~20km wide) zone of secondary contact where hybrids are produced and backcrossed with unadmixed individuals of the two parental species (Cortés-Ortiz et al., 2007; 2019). Because both species exhibit bi-sexual dispersal, it is unsurprising that the hybrid zone consists

of groups of individuals with different degrees of admixture. For example, some groups may have individuals from unadmixed parental species, as well as admixed recent generation hybrids to highly backcrossed hybrid individuals. Although some evidence of recent generation hybrids exists (Cortés-Ortiz et al., 2019), the research team has yet to find an F1 hybrid, indicating that they are very rare.

1.4 Introduction to this dissertation

In this dissertation, I expand on the hypothesis that there is more intense sperm competition in *A. palliata* compared to *A. pigra* by comparing sperm morphology between the two species. I expected that sperm traits would differ between the two species and that those differences would support the hypothesis that there is likely more intense sperm competition in *A. palliata* compared to *A. pigra*. I further characterized sperm morphology in multigenerational backcrossed hybrids from the hybrid zone, as well as proportions of morphologically abnormal sperm and testes, which could be the product of developmental deficiencies due to genomic admixture. My results support the hypothesis that there is likely stronger sperm competition in *A. palliata*. My results also suggest that genetic introgression from genes underlying these traits in *A. pigra* is more detrimental to *A. palliata*-like hybrid sperm and testes compared to the opposite.

Lastly, I compared putative sperm-related genes (i.e., genes known to be important for sperm development, function, and morphology in humans) between *A. palliata* and *A. pigra.* I found that most of these putative sperm-related genes in *A. palliata* and *A. pigra* are under purifying selection. I then identified Single Nucleotide Polymorphisms (SNP) in proximity to these genes from previous analysis of genome-wide SNP data already available for this hybrid system. I found that most of these genes have *A. palliata* ancestry. However, I did not find a unique pattern of reduced or increased introgression of all these SNPs, which would indicate

barrier or adaptive loci, respectively, in all putative sperm-related genes analyzed. This may indicate that not all genes of these genes are under the same selective pressures in the hybrid zone, perhaps because some may also be important for other genetic pathways. I also investigated the location of those genes in the human genome (because the howler monkey genomes are not annotated) and noted that some of these genes are located in what is known as human "deserts" of archaic ancestry, where the human genome is depleted of Neanderthal and Denisovan DNA (Sankararaman et al., 2016). In the *Alouatta* system, genes that were in the "deserts" of archaic ancestry in humans, mostly show excess *A. palliata* ancestry and reduced introgression, suggesting some of these autosomal loci may act similarly during speciation across primates. Some SNPs were also present in elevated regions of archaic ancestry and show mixed introgression patterns, suggesting further analysis with more genes is necessary. This work especially expands upon the evidence of shared X-linked loci in deserts of archaic ancestry that also contribute to reproductive isolation in this system (Baiz et al., 2020).

Overall, my dissertation contributes to the understanding of how inferred differences in sexual selection manifest in wild primate sperm morphology, as well as how hybridization affects sperm and introgression of putative sperm-related genes in a howler monkey hybrid zone. This work has opened more avenues for future speciation research in wild primates. Specifically, future testing of other sperm-related hypotheses, such as that sperm count should be higher and motility greater in *A. palliata* compared to *A. pigra,* would allow us to test consequences of the observed divergence more directly in sperm morphology between these species. The improvement of techniques during my fieldwork will be crucial to address this topic in future field expeditions. Similarly, my contribution of the first genome assembly for *A. pigra* now allows genomic comparisons between *A. palliata*, *A. pigra,* and hybrid individuals to uncover

genes and gene regions potentially associated with adaptive traits and with barriers to reproduction. In particular, whole-genome resequencing for individuals in the hybrid zone is now feasible and would provide the opportunity to further identify genes associated with sperm development and function, as well as other reproductive traits to analyze the extent to which they are involved maintaining species divergence in the face of hybridization. Additionally, my results in this howler monkey hybrid system will allow future comparative approaches testing the effects of hybridization on sperm between different hybridizing primate systems, such as the *Alouatta caraya* x *A. guariba clamitans* hybrid zones in Brazil and Argentina (Agostini, Holzmann, Di Bitetti, 2008; Bicca-Marques et al., 2008), and the *Papio spp.* baboon hybrid zones in Kenya and Zambia (e.g., Charpentier et al., 2012; Chiou et al., 2021), may reveal shared patterns of selection against male hybrids and hybridization across primates.

Chapter 2 : Differences in Sperm Morphology Between *Alouatta palliata* and *A. pigra* are Consistent with the Intensity of Sperm Competition in Each Species[1]

2.1 Abstract

The intensity of sperm competition, in which sperm compete within the female reproductive tract to reach and fertilize her eggs, varies in species with different mating systems. Sperm competition is more intense in species where males cannot monopolize access to reproductive females and females mate with multiple males. In this scenario, a morphological change that increases the ability of sperm to reach and fertilize eggs should rapidly spread in the population, leading to sperm morphological differences between closely related species. Differences in sperm morphology have been reported among primate species with different mating systems. However, due to the inherent logistical and ethical difficulties to sample sperm from wild primates, the extent of variation in sperm morphology within species and among closely related species remains understudied. Here, I compared sperm morphological traits from two sister howler monkey species (*Alouatta palliata* and *Alouatta pigra*) that have different mating systems to investigate the effect of sperm competition on sperm morphological traits. I predicted that sperm from *A. palliata*, where females have more opportunities to mate with multiple males, would show differences in traits associated with increase sperm competitiveness compared to A. pigra where females mostly mate with the central male. I used linear mixed models to

[1] This chapter is published as: Hirst MA, Rodas-Martínez AZ, Milich KM, Cortés-Ortiz L. (2023). Differences in sperm morphology between *Alouatta palliata* and *Alouatta pigra* are consistent with the intensity of sperm competition in each species. *Am. J. Primatol.* 85(10): e23538.

determine species differences in sperm morphology, controlling for individual variation. I found that midpieces and heads in *A. palliata* sperm were on average 26.2% and 11.0% longer, respectively, than those of *A. pigra*. Differences in these traits are important for sperm speed and hydrodynamic movement in other species and can affect fertilization success. This study provides empirical evidence of sperm morphological traits that evolved through sexual selection in sister primate species with different mating systems.

2.2 Introduction

Sperm are the most morphologically diverse animal cells (Lüpold & Pitnick, 2018) and can show remarkable diversity between closely related species (Omotoriogun et al., 2016; Kahrl & Cox, 2017; Gu et al., 2019) and even between populations (e.g., Kustra et al., 2019). In internally fertilizing species, sperm cells generally contain a head, which houses the male DNA and acrosomal enzymes for penetrating an egg, a midpiece containing mitochondria that provide energy to propel the cell in a progressive motion, and a tail that provides the mechanical means to move through the female reproductive tract. Sperm head shape and the lengths of the midpiece and tail evolve rapidly via sexual selection, and alterations in sperm traits may influence sperm cell reproductive success (Lüpold & Pitnick, 2018; Roldan, 2019; Fitzpatrick et al., 2020). For example, a male that produces sperm with longer midpieces may benefit from having more mitochondria, providing energy for those sperm cells to travel farther distances and/or at a greater speed than sperm from other males that have shorter sperm midpieces and fewer mitochondria (Anderson & Dixson, 2002; Anderson et al., 2005; Firman & Simmons, 2010a; 2010b; Piomboni et al., 2011; Fisher et al., 2016).

In species characterized by polygynandrous and polyandrous mating systems, females have opportunities to mate with multiple males during a given estrous period and post-copulatory

sexual selection such as sperm competition (i.e., sperm competing within the female reproductive tract to reach and fertilize her eggs, Parker, 1970) and cryptic female choice (i.e., the mechanisms that females exert to bias fertilization toward the sperm of specific males, Eberhard, 1996) are expected to be more intense. In species with such mating systems, males have larger testes relative to body size (i.e., the gonadosomatic index) compared to species characterized as polygynic or monogamous because selection favors greater sperm production for outcompeting sperm from other males in the female reproductive tract (Harcourt et al., 1981; Møller, 1988; Møller, 1989; Harcourt et al., 1995; Lüpold et al., 2020). Larger numbers of sperm may be reproductively advantageous by increasing the probability that a sperm will reach and fertilize an egg. Indeed, across taxa there is strong support for correlations between the male gonadosomatic index and mating system, such that polygynandrous mating systems tend to have larger male gonadosomatic index than polygynous or monogamous species (Short, 1979; Harcourt et al., 1981; Møller, 1988; Møller, 1989; Kelaita et al., 2011). However, less is known regarding the effect of selective pressures imposed by distinct mating systems on the morphology of sperm.

In primates, sperm morphology has been characterized in representative species of several families including Cercopithicidae, Lorisidae, and Lemuridae (Gould & Martin, 1978), Hominidae, Pongidae, and Cebidae (Martin, Gould, & Warner, 1975), and Callitrichidae and Atelidae (Steinberg et al., 2019; Valle et al., 2012; Valle et al., 2013a; 2013b; Swanson et al., 2016), illuminating the great diversity of sperm morphologies across primates. Recently, research has expanded into determining how and why sperm morphology differs between closely related primate species (Martinez & Garcia, 2020), yet such research is primarily focused on enhancing artificial reproductive technologies (e.g., in *Saimiri*, Sampaio et al., 2017; Comizzoli

& Holt 2022) or broadening the representation of primate species in the sperm morphometric literature (Steinberg et al., 2019). To better understand how divergent mating systems affect sperm morphology in closely related primate species, I compared sperm morphology between two endangered (IUCN, 2021) howler monkey sister species: the Mexican howler monkey, *Alouatta palliata mexicana* (henceforth *A. palliata*), and the Guatemalan black howler monkey, *A. pigra*.

Alouatta palliata and *A. pigra* diverged approximately 3 million years ago (Cortés-Ortiz et al., 2003) and differ in several life history traits including body size (Kelaita et al., 2011), social system and behavior (Treves, 2001; Van Belle et al., 2012; Ho et al., 2014; Baiz & Cortés-Ortiz, 2015), vocalizations (Kitchen et al., 2018), as well as in karyotype (Steinberg et al., 2014) and genetic diversity and structure (Cortés-Ortiz et al., 2003; Baiz et al., 2018; Melo-Carrillo, Dunn and Cortés-Ortiz, 2020). Importantly, *A. palliata* males have a larger gonadosomatic index than *A. pigra* males, suggesting that sexual selection via sperm competition is more intense in *A. palliata* than in *A. pigra* (Kelaita et al., 2011). This difference in gonadosomatic index aligns with the differences in mating systems between these species. Specifically, *A. palliata* are considered polygynandrous, with social groups consisting of multiple males, multiple females, and their offspring (Wang & Milton, 2003; Ryan et al., 2018). Both central (dominant) and non-central males successfully mate with receptive females (Jones, 1985; Corewyn, 2015), and females mate with multiple males even during the same estrous period (Jones & Cortés-Ortiz, 1998; Corewyn, 2015). In contrast, *A. pigra* are mostly polygynous, with social groups usually consisting of less than two adult males that may be related or not, one or more females, and their offspring (Bolin, 1981; Van Belle & Estrada, 2008; Van Belle, Estrada, Strier, 2008; Van Belle et al., 2009a). *Alouatta pigra* males are large, and central (dominant) males guard sexually

receptive females by keeping close proximity and forming strong social bonds with them (Van Belle et al., 2009a, 2009b), thus limiting access to potential male competitors. Mate guarding occurs in *A. palliata* as well, but because there are so many more males and females in each group (Corewyn, 2015), it is more difficult for *A. palliata* males to guard simultaneously receptive females (Van Belle & Bicca-Marques, 2015), thus giving females more opportunities to mate with different partners during a single estrous period (Jones, 1985; Jones & Cortés-Ortiz, 1998; Corewyn, 2015). *Alouatta pigra* females have been observed to engage in extra-pair copulations with subdominant wandering males, but central males have a higher likelihood of paternity (Van Belle & Estrada, 2008; Van Belle et al., 2014; Van Belle & Bicca-Marques, 2015). This pattern suggests a stronger role for pre-copulatory male competition in *A. pigra* groups, which may explain the smaller gonadosomatic index observed in this species (Kelaita et al., 2011).

It is unclear to what extent differences in the intensity of sperm competition in *A. palliata* and *A. pigra* affect sperm morphology; something that has not been studied in pairs of closely related primate species with distinct mating systems. Given the ~3-million-year (Cortés-Ortiz et al., 2003) divergence between these sister species and their differences in mating systems and gonadosomatic index (**Table 2-1**), I hypothesized that *A. palliata* sperm would have morphological traits that are associated with fast swimming speed in other taxa (Firman & Simmons, 2010b; Simpson et al., 2013), incuding longer midpieces. Sperm midpieces contain mitochondria in a stacked, cylindrical configuration along the length of the midpiece and have shown positive correlations between length and sperm swimming speed in different taxa (Amaral et al., 2013; Gu et al., 2019). Additionally, I expected to observe longer tails in *A. palliata* sperm because in other species (Lüpold et al., 2009; Firman & Simmons, 2010a; 2010b; Simpson et al.,

2013; Fisher et al., 2016) there is a positive correlation between sperm swimming speed and tail length while still maintaining the cell's overall hydrodynamic ability (Simpson et al., 2013; Bennison et al., 2016; Godwin et al., 2017; Losdat & Helfenstein, 2018).

2.3 Materials and Methods

2.3.1 Ethical Note and Data Availability

I completed all research described here in accordance with the University of Michigan Institutional Animal Care and Use Committee (PR00009319 and PRO00009558) and in adherence to the legal requirements in Mexico (SGPA/DGVS/03676/07, SGPA/DGVS/04949/07, SGPA/DGVS/03293/10, SGPA/DGVS/4936/19) and the United States (USFWS 08US178005/9, 12US46480A/9, 21US77243D/9). All research described here follows the American Society of Primatologists Ethical Principles for the Treatment of Non-Human Primates. All data used in this study are either presented in the supplemental materials or are available upon reasonable request.

2.3.2 Sampling

Between 2007 and 2019, our highly trained field team collected biological samples (blood and semen) from wild adult howler monkey males from two localities in Tabasco (*A. palliata*) and three from Campeche (*A. pigra*) in Mexico (**Figure 2-1**). We anesthetized, captured, and released all animals using the methods described in Kelaita et al. (2011) and collected sperm samples in accordance with the approved protocols and permits described in the Ethical Note. We determined the approximate age of each adult by observing dental development and wear patterns, as described in Kelaita et al. (2011). Briefly, we used dental features such as tooth eruption patterns and the amount of wear on the teeth to determine approximate categorical age

(i.e., infants, juveniles, subadults, adults). I only used sperm from adult individuals in my analyses.

Although *Alouatta palliata* and *A. pigra* hybridize in a narrow region in the state of Tabasco (Cortés-Ortiz et al., 2007; Cortés-Ortiz et al., 2019), and although most individuals in this study were sampled outside of the contact zone, three *A. palliata* individuals were sampled near the hybrid zone (**Figure 2-1** triangle where *A. palliata* has N = 3). These individuals were confirmed to be non-admixed using microsatellite (Cortés-Ortiz et al., 2019) and/or ddRAD sequencing data (Baiz et al., 2018). In total, I included samples from five *A. palliata* and eight *A. pigra* individuals for my analyses.

2.3.3 Semen collection and sperm slide preparation

All individuals were anesthetized with intramuscular ketamine hydrochloride before electrostimulation via an electroejaculator (Eletrojet®, Eletrovet, SP, Brazil) as described in Flores-Herrera et al. (2012) with minor modifications described below. Before beginning electroejaculation, I cleaned the penis and surrounding area with sterile gauze and water to avoid potential contamination. I gently inserted a 1 or 2.5-cm diameter rectal probe with three longitudinal copper electrodes (3 cm long) that was lubricated with sterile gel (K-Y Brand Jelly, Johnson & Johnson, New Brunswick, NJ, USA) into the rectum ~5 cm deep, with the three electrodes facing ventrally to maximize contact with the prostate. I administered up to two separate series of electrical stimuli to the prostate using the following protocol: initial stimulus of 2.0 V for 2 - 3 seconds, decreased back to 0 V, and subsequently increased by 0.5 - 1.0 V for 2 - 3 seconds. I continued this sequence until either ejaculation was achieved, 18 stimuli were administered, or a maximum of 10 V was reached. If no ejaculation was achieved after the first

series of electrical stimulus, I waited a few minutes before I started a new series to allow the monkey to rest. If after a second series the male did not ejaculate, I did not attempt a third series.

I collected ejaculate in a sterile tube and visually assessed the color and approximate volume. I made a preliminary evaluation of the ejaculate in the field using either a compound microscope (Swift F-31, Swift Instruments, International S. A., 2007 through 2010) or a phase-contrast microscope (CxL Binocular Cordless, LaboMed Inc., Los Angeles, CA, after 2010) to confirm sperm presence, estimate density, and define the dilution for preparing sperm smear slides. I used sperm washing medium (HTF; Irvine Scientific, Santa Ana, CA,) or Dulbecco's phosphate buffered saline (DPBS) solution (HyClone™ Laboratories, UT) to dilute the ejaculate. Dilutions vary from 1:10 to 1:200 according to sperm density. Two of the ejaculates had low volume, so the sample was used in its entirety for sperm smear slide preparation. I prepared two sperm smear slides per sample by mixing 10µL of the diluted sample with 10µL Eosin-nigrosin stain solution (Sigma-Aldrich). I air-dried sperm slides and later mounted them for preservation using Entellan® mounting media.

I photographed sperm from each slide using the phase-contrast microscope and a Digital 5MP Microscope Imager (Model 44421, Celestron LLC, Torrance, CA) (**Figure 2-2**) at 40X, 100X, 200X and 400X. I photographed at least 200 sperm for most individuals when possible, maximizing the probability of obtaining 100 sperm per individual for analysis. Actual sperm numbers obtained for measurement ranged from 30 to 100 with a median of 100 sperm (mean = 85.6 ± 23.8). To avoid photographing the same sperm twice, I visually located sperm along three transects on the slides. The first transect ran through the middle of the slide, and if there was a low number of spermatozoids, I followed a second and third transect along either side of the length of the slide. All slides are stored in the Rodas-Martínez lab at UJAT.

2.3.4 Sperm measurements

Using sperm photographs, I measured 100 mature, complete normal sperm cells per individual when possible (N = 500 sperm for *A. palliata,* N = 613 sperm for *A. pigra*). I defined mature, normal sperm cells as those with the three main sperm components intact (head, midpiece, and tail). Although I observed abnormal sperm in some of my slides, I did not measure sperm with abnormal morphology which I characterized as having a distorted head, midpiece or tail (World Health Organization, 2021) or had two heads or two tails. Additionally, I did not measure sperm that may have been physically distorted from the staining process, nor those for which I could not see the end of the sperm tail.

I used ImageJ® (Schneider et al., 2012) to measure ten pre-determined measurements of each sperm cell from the head, midpiece, and tail (**Figure 2-3**; World Health Organization, 2021; Steinberg et al., 2019; Swanson et al., 2016). WI categorized these as head length, head width, head circumference, midpiece length, midpiece width 1 (near the head), midpiece width 2 (in the middle), midpiece width 3 (near the tail), midpiece area, tail length, and total length. I used the straight-line tool to measure lengths, the elliptical tool to measure circumference, the polygon selections tool to measure area, and the segmented line tool for tail length and total length, which were rarely in a linear orientation.

2.3.5 Reliability of sperm morphology measurements

I took three approaches to ensure that my sperm morphology measurements were reliable and to account for intra-observer error. First, I designated a single person (MAH), who was blind to the previous measurements and the identity of the monkey, to do all the measuring. Second, I calculated reliability by measuring 20 random sperm from 3 random individuals of each species twice and ran t-tests between repeated measures. Third, I measured a single sperm before each

measuring session and used t-tests to compare new values with original values to ensure that measurements were not affected by time. Results from the t-tests for all but two variables were not significantly different in either analysis ($P>0.5$), suggesting those measurements are reliable. However, t-test analyses for repeated measures of tail length, and thus total length, differed significantly ($P<0.05$). Although I selected sperm for which I originally thought I could reliably identify the end of the tail, the significant differences in my repeated measurements could be due to common difficulties in accurately identifying the end of the tail in some sperm due to sample preparation. Changes in tail length were only in the order of 4.5±3.1 μm. Therefore, although I report these measurements, I only cautiously interpret them in the context of my discussion for comparisons with other platyrrhines.

2.3.6 Statistical analyses to determine differences in sperm traits between species

To determine the differences between sperm morphological traits between *A. palliata* and *A. pigra*, I first assessed the distribution of sperm measurements in RStudio version 1.4.1103 (RStudio Team, 2020) and visualized the normality of the raw data using Q-Q plots. Additionally, I checked for any violation of these assumptions by plotting histograms of the residuals for each model and verified that residuals were close to a normal distribution. I visualized the spread of the data with violin plots to explore sperm morphology variation in individuals and between species. Due to the large extent of variation observed per individual, I used linear mixed models to control for intra-individual variation. The linear mixed models showed that all residuals were centered around zero, suggesting that the models are a good fit for the data.

I used the *lmer* function in the *lme4* package (Bates et al., 2015) in RStudio to fit linear mixed models using restricted maximum-likelihood by setting each sperm measurement as the

outcome variable, species as the fixed effect, and individual identity as a random effect. This approach allowed us to hold individual variation constant to control for correlational error while the model determined how species identity affected each sperm measurement. I extracted confidence intervals for the model parameters and noted for each measurement and species where the 5% and 95% confidence intervals did not cross zero (i.e., two negative values or two positive values) to determine significant associations between species and sperm morphological traits.

2.3.7 Evaluation of the proportion of normal versus abnormal sperm

I quantified the proportion of abnormal sperm between *A. palliata* and *A. pigra* because selection is expected to favor high-quality sperm production in species with more intense sperm competition (e.g., Pitnick et al., 2009; van der Horst & Maree 2013). I counted 50 sperm cells per individual and categorized each as either normal or abnormal based on the WHO laboratory manual for evaluating human semen (World Health Organization, 2021). My dataset consisted of counts of normal and abnormal sperm for individuals from both species where each row represented a sperm cell from an individual, with information on species identity (fixed effect) and whether or not that sperm was normal or abnormal (outcome variable). I fitted a binomial generalized mixed effect model (GLMM) in R version 4.3.0 using the glmer() function in the lme4 package (Bates et al., 2015) to evaluate the effects of species on sperm abnormality. The binomial outcome variable in my GLMM was whether or not each sperm cell for each individual was abnormal (1) or normal (0), the fixed effect was species identity, and I accounted for individual variation within species by incorporating individual identity as a random effect. I calculated odds ratios (OR) to interpret the results of my model.

2.4 Results

As predicted, species identity was a significant predictor of sperm midpiece length (β = -0.42, 95% CI [-0.66, -0.19], **Table 2-2, Figure 2-3**), with *A. palliata* sperm being 26.2% longer on average than *A. pigra* sperm. My analyses of sperm tail length did not reveal any statistically significant differences between the two species (β = -0.32, 95% CI [-1.65, 1.01], **Table 2-2**), but due to the inconsistencies in my own measuring of this trait, I was unable to reliably use these results to test my prediction. Species identity was also a significant predictor of sperm head length (β = -0.21, 95% CI [-0.36, -0.06], **Table 2-2**) with *A. palliata* sperm heads being 11.0% longer on average than those in *A. pigra* (**Figure 2-3**). For the sperm midpiece length, the variance term from my model showed that 31% of the variation was due to individual variation, and 69% was due to differences between species, and for the sperm head length, 27% of the variation was due to individual variation and the remaining 73% was due to differences between species. I did not observe significant effect size differences in any of the other sperm measurements between species (**Table 2-2, Table 2-3** for means of each sperm measurement per howler monkey individual).

2.4.1 Sperm abnormalities

I examined a total of 250 *A. palliata* sperm from 5 individuals and 400 *A. pigra* sperm from 8 individuals and categorized each sperm cell as either normal or abnormal. Overall, I found that *A. palliata* individuals had 6.8% (17/250) abnormal sperm, while *A. pigra* had 19.8% (79/400) abnormal sperm. To determine if species identity affected abnormal sperm count, I fitted a binomial GLMM that accounted for individual variation and found a statistically significant difference in sperm abnormalities between species. The results of my model showed that *A. pigra* males were three times more likely to have abnormal sperm compared to *A. palliata*

(β = 1.11, 95% CI_{β} [0.36, 1.95], OR = 3.03, 95% CI_{OR} [1.43, 7.00]). The random effects intercept showed a variance of 0.22 ± 0.47, representing low variability among individuals within each species.

2.5 Discussion

Sperm morphology is an important but difficult to study metric associated with intrasexual selection, cryptic female choice, and sexual conflict (Stumpf et al., 2011). My project contributes novel data from two howler monkey sister species with differing mating systems that can illustrate the effect of sexual selection on sperm morphology. I found that sperm midpiece and head length differ significantly between *A. palliata* and *A. pigra*, and that both measurements are longer in *A. palliata*. Because *A. palliata* is characterized by a polygynandrous mating system and males have a high gonadosomatic index, it has been hypothesized that this species exhibits a higher intensity of sperm competition than *A. pigra* (Kelaita et al., 2011). My results support this hypothesis and the prediction that species in which females have multiple partners would have longer sperm midpieces that help propel the sperm to reach the egg faster. I was unable to test the prediction that sperm tail length would differ between two species because my sperm tail measurements were not fully accurate.

2.5.1 Why are sperm midpiece and head longer in A. palliata*?*

Positive correlations between mating system and sperm midpiece size are widespread in captive primates, where larger sperm midpieces are reported in species where females have multiple mating partners (Anderson & Dixson, 2002; Anderson et al., 2005; Martínez & García, 2020). Sperm midpieces are packed with energy-producing mitochondria, which are helically stacked along the axoneme and provide energy to propel the flagellum (Fawcett, 1970; Olson & Winfrey,

1990; Gu et al., 2019). Thus, longer sperm midpieces should contain more mitochondria, resulting in faster sperm or sperm with greater endurance (e.g., Gage, 1998; Piomboni et al., 2011; Gu et al., 2019; Comizzoli & Holt, 2022). This pattern is supported by a study that combined micro- and macro-sperm morphology with sperm swimming speed and adenosine triphosphate (ATP) content in sperm of several mammalian species (Gu et al., 2019). ATP content assays combined with three-dimensional internal ultrastructure analysis of mitochondria revealed positive correlations between the volume of mitochondria, ATP content, and sperm swimming speed (Gu et al., 2019). Sperm midpiece length was strongly associated with mitochondrial volume and sperm swimming speed across the species analyzed (Gu et al., 2019). If these patterns extend to the howler monkey study system, then my hypothesis that the more intense sperm competition in *A. palliata* may have resulted in sperm with longer midpieces, and thus faster swimming sperm is supported. A comparative analysis of sperm swimming speed and sperm midpiece length between *A. palliata* and *A. pigra* would help reveal the relationship between these traits. Unfortunately, sampling conditions, including lack of proper equipment, across several years and expeditions prevented us from consistently collecting data on sperm swimming speed from my samples.

Ratios between sperm component lengths, such as midpiece length to total length, have been associated with greater sperm swimming speed in several taxa (Tourmente et al., 2011, Temple-Smith, Ravichandran, Nunez, 2018). These relationships may occur because longer flagella tend to be thicker in diameter and thus require more energy to move properly (Gu et al., 2019) or because large sperm heads relative to tail length may induce drag and hinder sperm motility (e.g., Temple-Smith, Ravichandran, Nunez, 2018). I created a repeated measures correlation matrix between all sperm measurements (e.g., head and tail lengths, head and

midpiece lengths, head, and total lengths, etc.) and found no significant correlations (**Table 2-4).** The factors regulating midpiece and flagellum size are poorly known in general, but the outer dense fibers of the sperm tail and the size of mitochondria may correlate with flagellar length in some animals (Gu et al., 2019). If this correlation was true in the species analyzed in this study, I would expect to see longer flagella in *A. palliata* compared to *A. pigra* and a correlation between midpiece length and tail length. However, given the difficulty in accurately measure tail lengths in the sample, the assessment of this correlation remains to be analyzed with more powerful technologies (e.g., electron microscopy).

Across animals, sperm head generally varies in shape to accommodate the organization of the nucleus and acrosome of the cell (Mortimer, 2018). Specific sperm head morphologies have been associated with sperm swimming speed in birds (Støstad et al., 2018), fish (Fitzpatrick et al., 2009), and mammals (Malo et al., 2006; Tourmente et al., 2011; Varea Sánchez et al., 2013; García-Vázquez et al., 2016; Varea-Sánchez et al., 2016). Although sperm head characteristics greatly vary across taxa, sperm head elongation tends to correlate with faster swimming speed (e.g., Malo et al., 2006; Støstad et al., 2018; but see Rowe et al., 2015), for example by reducing drag while moving through the female reproductive tract. In reproductively active red deer, there is a positive correlation between sperm head length and sperm speed (Malo et al., 2006). In the primate species I analyzed, head length is significantly longer in *A. palliata* than *A. pigra,* consistent with the hypothesis that sperm swimming speed may be greater in *A. palliata* because of the selection pressure for faster sperm that can more likely reach and fertilize the egg.

The proportion of normal to abnormal sperm can reflect the intensity of sperm competition (e.g., van der Horst & Maree, 2013; Pitnick et al., 2009) because selection acts

against abnormally shaped sperm that may function poorly and hinder fertilization. I found that the odds of observing abnormal sperm in *A. pigra* were three times higher compared to *A. palliata*. These findings demonstrate a substantial difference in the number of abnormal sperm cells between these species, even after accounting for individual variation. This finding aligns with my hypothesis that *A. palliata* are under more intense sexual selection pressure to produce more efficient sperm than *A. pigra*.

2.5.2 Is sperm length constrained in **Alouatta***?*

In several taxa with internal fertilization, longer sperm in general are positively correlated with increased swimming speed and competitiveness (e.g., across invertebrates and vertebrates, Kahrl et al., 2021; in beetles, Godwin et al., 2017; and in birds, Lüpold et al., 2009; Losdat & Helfenstein, 2018; Støstad et al., 2018). Although my results imply that some morphological traits (longer midpieces and heads) known to increase sperm speed are selected for in *A. palliata*, it is possible that overall sperm length, which is also associated with increasing swimming speed, might be constrained by other factors. For example, there is a known tradeoff between sperm size and number (Parker, 1982; Parker, 1993; Parker & Pizzari, 2010; Immler et al., 2011), where producing many small sperm might sometimes be more adaptive than the opposite. There is evidence that selection may favor sperm number over sperm size in large mammal species (Lüpold & Fitzpatrick, 2015), and howler monkeys are amongst the largest platyrrhine primates.

Sperm morphology has only been characterized for one other howler monkey species (*Alouatta caraya*) (Valle et al., 2004; Moreland et al., 2011; Valle et al., 2013a; 2013b; Carvalho et al., 2014; Swanson et al., 2016; Steinberg et al., 2019; Martinez & Garcia, 2020). Like *A. palliata, A. caraya* has a polygynandrous mating systems and a positive correlation between mean number of males and testes volume (Dunn et al., 2015), suggesting some degree of sperm

competition, although formal analyses of the gonadosomatic index have not been reported. Comparisons of my measures of sperm length between *A. palliata, A. pigra,* and published *A. caraya* data (52.01 µm, Steinberg et al., 2019) suggest similar mean total lengths among these howler monkeys. Although my measurements of sperm length may not be completely accurate for all the sperm in the sample considering the level of variation across my repeated measurements, I deemed that these measurements may still reflect the overall sperm length for *Alouatta* relative to other taxa. Comparisons of sperm length with other platyrrhine primates show that *Alouatta* sperm are generally on the shorter end compared to other platyrrhine species (**Table 2-5;** Swanson et al., 2016; Steinberg et al., 2019). In particular, the mean total sperm lengths of these three *Alouatta* species are more on par with those observed among monogamous or polyandrous species (e.g., *Aotus lemurinus*, *Callithrix jacchus*, and *Saguinus* sp.) and much shorter than those in *Ateles*, in which males and females mate with multiple partners (Campbell, 2006; Gibson et al, 2007) (**Table 2-5**). This pattern could suggest that the overall length of sperm might be phylogenetically constrained in *Alouatta*, regardless of the mating system.

I did not find significant differences in the remaining sperm morphological traits that I measured. These findings are consistent with my prediction that there might be constraints on sperm morphology to foster proper hydrodynamic propulsion, reducing energetically costly drag, and supporting progressive motion (e.g., Humphries et al., 2008). Although sperm competition is known to place strong selective pressure on sperm morphology, it is necessary to recognize that sperm competition may not be the only factor driving sperm morphological evolution in these species. Cryptic female choice (Eberhard, 1990; Eberhard, 2009; Firman et al., 2017), sexual conflict (Parker & Partridge, 1998; Rowe et al., 2020), and subsequent male-female coevolution (Eberhard, 2009), as well as eco-evolutionary dynamics (Svensson, 2018) likely also play a role.

Here, I interpreted my results focusing on the effect of sperm competition in shaping sperm morphology. The extent to which sexual conflict and cryptic female choice affect sperm morphological traits remains unknown (Firman et al., 2017), but it is plausible that it could affect sperm morphology in ways that are beyond those specifically affecting swimming speed and motility. However, the difficulties in studying reproductive tissues from wild female howler monkeys limit the ability to investigate these evolutionary mechanisms. Nonetheless, future studies on howler monkey reproductive evolution should also consider aspects such as the female reproductive microbiome and the evolution and expression of genes known to be involved in mechanisms of cryptic female choice in other species (Firman et al., 2017). Additionally, analyzing sperm morphology in the well-characterized hybrid zone between *A. palliata* and *A. pigra* in Tabasco, Mexico (Cortés-Ortiz et al., 2007; Baiz et al., 2018) could provide the means to understand the potential adaptive value of the sperm morphological differences I observed in this study. If the longer sperm head and midpiece lengths typical of *A. palliata* are present in admixed individuals that are otherwise more genetically like *A. pigra*, it would imply adaptive introgression of genes associated with these features.

2.6 Conclusion

This study is the first to report sperm morphological traits in *A. palliata* and *A. pigra*, two endangered primate taxa, and one of the first studies to analyze sperm from wild platyrrhine primates (but see Sampaio et al., 2017 and Oliveira et al., 2016). My results provide additional support for the hypothesis that stronger sperm competition occurs in *A. palliata* compared to *A. pigra* (Kelaita et al., 2011) and that *A. palliata* sperm have traits associated with faster swimming abilities than *A. pigra* sperm, which could give them a competitive advantage to reach and

fertilize a female's ovum. Overall, this study adds to the understanding of the effect of sexual selection on sperm morphological traits in primates with different mating systems.

Table 2-1: Summary of relevant *Alouatta palliata* and *A. pigra* sexually selected traits and predicted sperm morphology traits.

Gonadosomatic index is calculated here as average testes volume (cm^3)/average body mass (g)x100. In between brackets are the averages and standard deviations for each measurement.

Species	Gonadosomatic index (Testis volume, body weight, Kelaita et al., 2011)	Mating system	Inferred sperm competition (Kelaita et al., 2011)	Predicted sperm morphology
Alouatta palliata	0.39 (22.7 ± 10.9 cm^3, 5800 ± 690 g)	Polygynandrous (Ryan et al., 2008; Wang & Milton 2003)	More intense	Longer midpiece, longer tail
Alouatta pigra	0.15 (11.3 ± 3.8 cm^3, 7600 ± 1130 g)	Mostly polygynous (Bolin, 1981; Van Belle et al., 2008, 2009)	Less intense	Shorter midpiece, shorter tail

Table 2-1 References:

- Bolin, I. (1981). Male parental behavior in black howler monkeys (*Alouatta palliata pigra*) in Belize and Guatemala. *Primates*, *22*(3), 349-360. https://doi.org/10.1007/BF02381575
- Kelaita, M., Dias, P. A. D., Aguilar-Cucurachi, M. S., Canales-Espinosa, D., & Cortés-Ortiz, L. (2011). Impact of intrasexual selection on sexual dimorphism and testes size in the Mexican howler monkeys *Alouatta palliata* and *A. pigra*. *American Journal of Physical Anthropology*, *146*(2), 179-187. https://doi.org/10.1002/ajpa.21559
- Ryan, S. J., Starks, P. T., Milton, K., Getz, W. M. (2008). Intersexual conflict and group size in *Alouatta palliata*: a 23-year evaluation. *International Journal of Primatology*, *29*: 405-420. https://doi.org/10.1007/s10764-007-9172-2
- Van Belle, S., & Estrada, A. (2008). Group size and composition influence male and female reproductive success in black howler monkeys (*Alouatta pigra*). *American Journal of Primatology*, *70*(6), 613-619. https://doi.org/10.1002/ajp.20534
- Van Belle, S., Estrada, A., Ziegler, T. E., & Strier, K. B. (2009). Sexual behavior across ovarian cycles in wild black howler monkeys (*Alouatta pigra*): male mate guarding and female mate choice. *American Journal of Primatology. 71*(2), 153-164. https://doi.org/10.1002/ajp.20635
- Wang, E., & Milton, K. (2003). Intragroup social relationships of male *Alouatta palliata* on Barro Colorado Island, Republic of Panama. *International Journal of Primatology*, *24*, 1227-1243. https://doi.org/10.1023/B:IJOP.0000005989.29238.ce

Table 2-2: Model estimates of fixed and random effects using raw data.

Note: The confidence intervals that indicate significance are shown in bold.
Abbreviations: SD, standard deviation; Var., variance.

Parameter	Point Estimate (β)	Standard Error	Lower-95	Upper-95	Random Effect (Var.)	Random Effect (SD)	Residual (Var.)	Residual (SD)
Head Length	-0.21	0.08	**-0.36**	**-0.06**	0.02	0.13	0.05	0.22
Head Width	-0.05	0.10	-0.24	0.14	0.03	0.17	0.03	0.18
Head Circumference	0.01	0.01	-0.01	0.03	0.0003	0.02	0.00099	0.03
Midpiece Length	-0.42	0.12	**-0.66**	**-0.19**	0.04	0.21	0.10	0.31
Midpiece Width 1	-0.01	0.05	-0.12	0.10	0.01	0.10	0.01	0.12
Midpiece Width 2	-0.03	0.05	-0.12	0.07	0.01	0.08	0.01	0.11
Midpiece Width 3	-0.02	0.03	-0.08	0.05	0.003	0.06	0.01	0.07
Midpiece Area	14.2	13.5	-12.1	40.5	558	23.6	257	16.0
Tail Length	-0.32	0.68	-1.65	1.01	1.39	1.18	4.0	2.00
Total Length	-0.90	0.79	-2.44	0.65	1.89	1.37	4.5	2.10

Table 2-3: Mean sperm measurements for each individual.

Individual identification numbers were assigned at capture in the field. Each mean is based on the number of sperm measured, which ranges from 30 to 100 with a median of 100 sperm (mean = 85.6 ± 23.8). Measurements that are significantly different between species are in bold.

Species	Individual ID	Number of sperm measured	**Mean head length (µm)**	Mean head width (µm)	Mean head circumference (µm)	**Mean midpiece length (µm)**	Mean midpiece width 1 (µm)	Mean midpiece width 2 (µm)	Mean midpiece width 3 (µm)	Mean midpiece area (μm^2)	Mean tail length (µm)	Mean total length (µm)
A. palliata	69-02	100	**6.72**	3.66	2.70	**4.76**	1.34	1.05	0.66	433	39.8	51.2
A. palliata	77-01	100	**6.42**	4.53	2.82	**5.23**	1.35	1.13	0.69	344	41.3	53.2
A. palliata	77-03	100	**6.54**	4.44	2.79	**5.46**	1.38	1.14	0.64	411	44.6	56.9
A. palliata	88-02	100	**6.48**	4.86	2.84	**6.42**	2.00	1.72	1.04	214	46.1	59.2
A. palliata	88-03	100	**6.90**	4.98	2.85	**6.09**	2.00	1.79	1.13	298	47.8	60.8
A. pigra	27-01	58	**5.77**	4.24	2.86	**3.98**	1.50	1.14	0.74	405	43.2	53.1
A. pigra	29-3	100	**5.68**	4.56	2.91	**3.81**	1.54	1.24	0.74	316	44.0	53.4
A. pigra	30-1	59	**5.68**	4.28	2.81	**4.99**	1.75	1.37	0.74	455	47.2	58.8
A. pigra	30-3	66	**6.63**	4.43	2.78	**4.15**	1.51	1.19	0.71	425	48.0	59.0
A. pigra	31-1	100	**6.67**	4.94	2.86	**5.49**	2.06	1.62	1.04	323	42.8	55.1
A. pigra	32-1	30	**5.82**	4.69	2.89	**4.45**	1.40	1.27	0.69	422	38.0	48.8
A. pigra	33-01	100	**5.63**	4.39	2.88	**3.93**	1.59	1.34	0.84	306	43.1	53.8
A. pigra	67-02	100	**5.37**	3.24	2.79	**3.85**	1.25	1.07	0.64	412	37.3	46.7

Table 2-4: Repeated measures correlation matrix between sperm measurements within individuals.

Using the rmcorr* package in R, I set participant as individual, measure1 as sperm measurement, and measure2 as the next sperm measurement. Statistically significant correlations are shown in bold; however, the biological significance of these specific correlations is not relevant because they are all within sperm components, and not across sperm components.

	Head Length	Head Width	Head Circumference	Midpiece Length	Midpiece Area	Midpiece Width 1	Midpiece Width 2	Midpiece Width 3	Tail Length	Total Length
Head Length										
Head Width	**0.37**									
Head Circumference	-0.26	**0.42**								
Midpiece Length	0.15	0.07	0.02							
Midpiece Area	0.00	-0.10	-0.07	0.07						
Midpiece Width 1	0.12	0.25	0.09	0.21	-0.12					
Midpiece Width 2	0.13	0.22	0.08	0.22	-0.07	**0.60**				
Midpiece Width 3	0.14	0.18	0.02	0.17	-0.05	**0.42**	**0.53**			
Tail Length	0.14	0.11	0.03	0.13	-0.03	0.13	0.15	0.05		
Total Length	0.23	0.14	0.00	0.26	-0.02	0.17	0.19	0.09	**0.95**	

*Bakdash JZ, Marusich LR. (2017). Repeated Measures Correlation. *Front. Psychol.* 8: 456. https://doi.org/10.3389/fpsyg.2017.00456

Table 2-5: Average sperm total lengths, midpiece lengths, and head lengths of diverse primates from published sources.

Species	N individuals	Captive / wild	# Sperm	Average sperm length (µm)	Average midpiece length (µm)	Average head length (µm)	References
Alouatta caraya	2	C	NA	50.1	3.49	4.94	g
Alouatta caraya	5	C	2500	NA	NA	4.66	h
Alouatta caraya	5	C	NA	NA	NA	4.66	i
Aotus lemurinus	3	C	631	55.1	6.6	2.8	e
Ateles belzebuth	1	C	NA	70.83	6.56	5.2	g
Ateles chamek	1	C	NA	64.09	7.34	5.16	g
Ateles geoffroyi	5	C	2500	NA	NA	4.51	h
Ateles paniscus	NA	C	NA	NA	10.2	6.4	a
Ateles paniscus	NA	C	NA	65.1	NA	NA	c
Callimico goeldii	5	C	NA	NA	NA	4.95	j
Callithrix jacchus	5	C	2500	NA	NA	5.19	h
Callithrix jacchus	NA	C	NA	NA	3.9	5.6	a
Callithrix jacchus	NA	C	NA	52.1	NA	NA	c
Callithrix pygmaea	NA	C	NA	NA	8.5	4.6	a
Callithrix pygmaea	NA	C	NA	54	NA	NA	c
Cebus albifrons	NA	C	NA	NA	13.2	9	a
Cebus albifrons	NA	C	NA	80.4	NA	NA	c
Cebus apella	NA	C	NA	NA	11.6	8.5	a
Cebus cay	3	C	NA	65.06	8.67	6.14	g
Cebus goeldii	5	C	2500	NA	NA	4.95	h
Cebus nigritus	1	C	NA	66.27	9.35	6.29	g
Cercocebus galeritus	NA	C	NA	93.1	NA	NA	c
Cercopithecus aethiops	NA	C	96	66.73	10.18	5.22	d
Cercopithecus nictitans	NA	C	NA	NA	12.3	5.3	a
Cercopithecus nictitans	NA	C	NA	58.1	NA	NA	c
Cercopithecus petaurista	NA	C	NA	NA	11.2	6.3	a
Cercopithecus petaurista	NA	C	NA	36.4	NA	NA	c
Chlorocebus aethiops	NA	C	NA	NA	12.1	6.2	a
Chlorocebus pygerythrus	NA	C	NA	88	NA	NA	c
Daubentonia madagascariensis	NA	C	NA	55.8	NA	NA	c
Erythrocebus patas	NA	C	NA	NA	14.5	7.8	a
Erythrocebus patas	NA	C	NA	89.5	NA	NA	c
Eulemur fulvus	NA	C	NA	NA	11	5.6	a
Eulemur macaco	NA	C	NA	NA	10.9	4.8	a
Eulemur mongoz	NA	C	NA	NA	13.5	6.2	a
Eulemur mongoz	NA	C	NA	62.9	NA	NA	c
Galago crassicaudatus	NA	C	63	60.3	9.5	5.6	b
Galago senegalensis	NA	C	NA	NA	11.6	3.9	a
Galago senegalensis	NA	C	75	61.2	9	4.9	b

Galago senegalensis	NA	C	NA	61.2	NA	NA	c
Gorilla gorilla	15	C	NA	NA	13.2	6.8	a
Gorilla gorilla	NA	C	94	61.17	11.74	9.78	d
Homo sapiens	NA	NA	NA	NA	5.9	4.5	a
Homo sapiens	NA	NA	99	58.39	4.7	6.11	d
Hylobates lar	NA	C	NA	NA	8.9	7.2	a
Hylobates lar	NA	C	NA	63.5	NA	NA	c
Hylobates syndactylus	NA	C	NA	NA	8.2	7.5	a
Lemur catta	NA	C	NA	NA	13.2	4.4	a
Lemur catta	NA	C	94	57.8	10.3	5.2	b
Lemur macaco	NA	C	115	54.9	10.6	7.1	b
Lemur variegatus	NA	C	49	104.9	20.7	7.5	b
Lepilemur ankaranensis	NA	C	NA	52	NA	NA	c
Lophocebus albigena	NA	C	NA	NA	11.1	5.8	a
Lophocebus albigena	NA	C	NA	34.2	NA	NA	c
Macaca arctoides	NA	C	NA	NA	10.5	6.8	a
Macaca arctoides	NA	C	98	77.61	10.53	5.57	d
Macaca fascicularis	NA	C	NA	NA	11.4	6.1	a
Macaca fascicularis	NA	C	100	73.63	11.06	5.53	d
Macaca mulatta	NA	C	NA	NA	11.1	5.3	a
Macaca mulatta	NA	C	108	73.83	11.06	5.31	d
Macaca mulatta	NA	C	NA	74.1	NA	NA	c
Macaca nemestrina	NA	C	NA	90.4	NA	NA	c
Macaca nemsetrina	NA	C	NA	NA	13.7	7.4	a
Macaca silenus	NA	C	NA	NA	9.8	6.7	a
Macaca silenus	NA	C	NA	32.9	NA	NA	c
Macaca sinica	NA	C	NA	69.1	NA	NA	c
Macaca sylvanus	NA	C	NA	NA	12.8	5.5	a
Macaca sylvanus	NA	C	NA	80.4	NA	NA	c
Macaca thibetana	NA	C	NA	73.7	NA	NA	c
Mandrillus leucophaeus	NA	C	NA	NA	15.2	5	a
Mandrillus leucophaeus	NA	C	NA	35.8	NA	NA	c
Mandrillus sphinx	NA	C	NA	NA	17.1	6.8	a
Mandrillus sphinx	NA	C	71	71.5	10.2	5.7	b
Microcebus murinus	NA	C	NA	NA	13.7	6.5	a
Microcebus murinus	NA	C	NA	75.2	NA	NA	c
Microcebus myoxinus	NA	C	NA	93.6	NA	NA	c
Nycticebus coucang	NA	C	NA	NA	14	5.1	a
Nycticebus coucang	NA	C	42	90	13.5	8.5	b
Nycticebus coucang	NA	C	NA	91	NA	NA	c
Pan paniscus	NA	C	NA	NA	8.9	4.7	a
Pan paniscus	NA	C	NA	68.1	NA	NA	c
Pan troglodytes	NA	C	NA	NA	6.3	4.7	a
Pan troglodytes	NA	C	106	57.36	6.34	4.68	d
Papio anubis	NA	C	NA	NA	8.8	4.9	a
Papio anubis	NA	C	NA	73	NA	NA	c
Papio cynocephalus	NA	C	NA	NA	10.3	4.6	a
Papio cynocephalus	NA	C	98	76.2	10.3	4.6	b
Papio cynocephalus	NA	C	98	76.19	10.3	4.62	d
Papio hamadryas	NA	C	NA	NA	12.7	6.5	a

Papio hamadryas	NA	C	NA	77.2	NA	NA	c
Pongo pygmaeus	NA	C	NA	NA	8.9	4.9	a
Pongo pygmaeus	NA	C	102	66.58	9.01	5.31	d
Pygathrix namaeus	NA	C	NA	NA	11.5	4.3	a
Pygathrix nemaeus	NA	C	NA	39.3	NA	NA	c
Saguinus midas	NA	C	NA	54.6	NA	NA	c
Saguinus midas	NA	C	NA	NA	9.1	5.3	a
Saguinus oedipus	NA	C	NA	54.8	NA	NA	c
Saguinus oedipus	NA	C	NA	NA	11.2	4.7	a
Saimiri boliviensis	1	C	205	76.8	10.5	4.3	e
Saimiri collinsi	10	C	NA	76.7	NA	NA	f
Saimiri sciureus	NA	C	NA	NA	10.1	5.6	a
Saimiri sciureus	NA	C	107	69.24	9.03	5.11	d
Saimiri sciureus	NA	C	NA	71.1	NA	NA	c
Saimiri vanzolinii	2	W	NA	76.1	NA	NA	f
Sapajus apella	NA	C	NA	84.1	NA	NA	c
Symphalangus syndactylus	NA	C	NA	68.9	NA	NA	c
Theropithecus gelada	NA	C	NA	NA	12.3	6.8	a
Theropithecus gelada	NA	C	NA	87.3	NA	NA	c

NA = data not available

Table 2-5 References:

a. Anderson, M. J., Nyholt, J., & Dixson, A. F. (2005). Sperm competition and the evolution of sperm midpiece volume in mammals. *Journal of Zoology. 267*(2), 135-142. https://doi.org/10.1017/S0952836905007284

b. Gould, K. G., & Martin, D. E. (1978). Comparative morphology of primate spermatozoa using scanning electron microscopy. II. Families Cercopithecidae, Lorisidae, Lemuridae. *Journal of Human Evolution. 7*(7), 637-642. https://doi.org/10.1016/S0047-2484(78)80048-7

c. Kahrl, A. F., Snook, R. R., & Fitzpatrick, J. L. (2021). Fertilization mode drives sperm length evolution across the animal tree of life. *Nature Ecology & Evolution, 5*(8), 1153-1164. https://doi.org/10.1038/s41559-021-01488-y

d. Martin, D. E., Gould, K. G., Warner, H. (1975). Comparative morphology of primate spermatozoa using scanning electron microscopy. I. Families Hominidae, Pongidae, Cercopithecidae and Cebidae. *Journal of Human Evolution. 4*, 287-292. https://doi.org/10.1016/0047-2484(75)90067-6

e. Nakazato C, et al. (2015). Morphological characterization of spermatozoa of the night monkey. *J. Mamm. Ova Res.* 32(1): 37-40. https://doi.org/10.1274/jmor.32.37

f. Sampaio, W. V., Oliveira, K. G., Leão, D. L., Caldas-Bussiere, M. C., Queiroz, H. L., Paim, F. P., Santos, R. R., & Domingues, S. F. S. (2017). Morphologic analysis of sperm from two neotropical primate species: comparisons between the squirrel monkeys *Saimiri collinsi* and *Saimiri vanzolinii*. *Zygote, 25*(2), 141-148. https://doi.org/10.1017/s0967199416000411

g. Steinberg, E. R., Sestelo, A. J., Ceballos, M. B., Wagner, V., Palermo, A. M., & Mudry, M. D. (2019). Sperm morphology in neotropical primates. *Animals: An Open Access Journal from MDPI, 9*(10), 839. https://doi.org/10.3390/ani9100839

h. Swanson, W., Valle, R., Carvalho, F., Arakaki, P., Rodas-Martínez, A., Muniz, J., & García-Herreros, M. (2016). Sperm morphology assessment in captive neotropical primates. *Reproduction in Domestic Animals, 51*(4), 623-627.

https://doi.org/10.1111/rda.12711

i. Valle, R. R., Carvalho, F. M., Muniz, J. A., Leal, C. L., & García-Herreros, M. (2013a). Differential distribution of sperm subpopulations and incidence of pleiomorphisms in ejaculates of captive howling monkeys (*Alouatta caraya*). *Naturwissenschaften, 100*(10), 923-933. https://doi.org/10.1007/s00114-013-1092-8

j. Valle, R. R., Arakaki, P. R., Carvalho, F. M., Muniz, J. A., Leal, C. L., & García-Herreros, M. (2013b). Identification of sperm head subpopulations with defined pleiomorphic characteristics in ejaculates of captive Goeldi's monkeys (*Callimico goeldii*). *Animal Reproduction Science. 137*(1-2), 93-102. https://doi.org/10.1016/j.anireprosci.2012.12.007

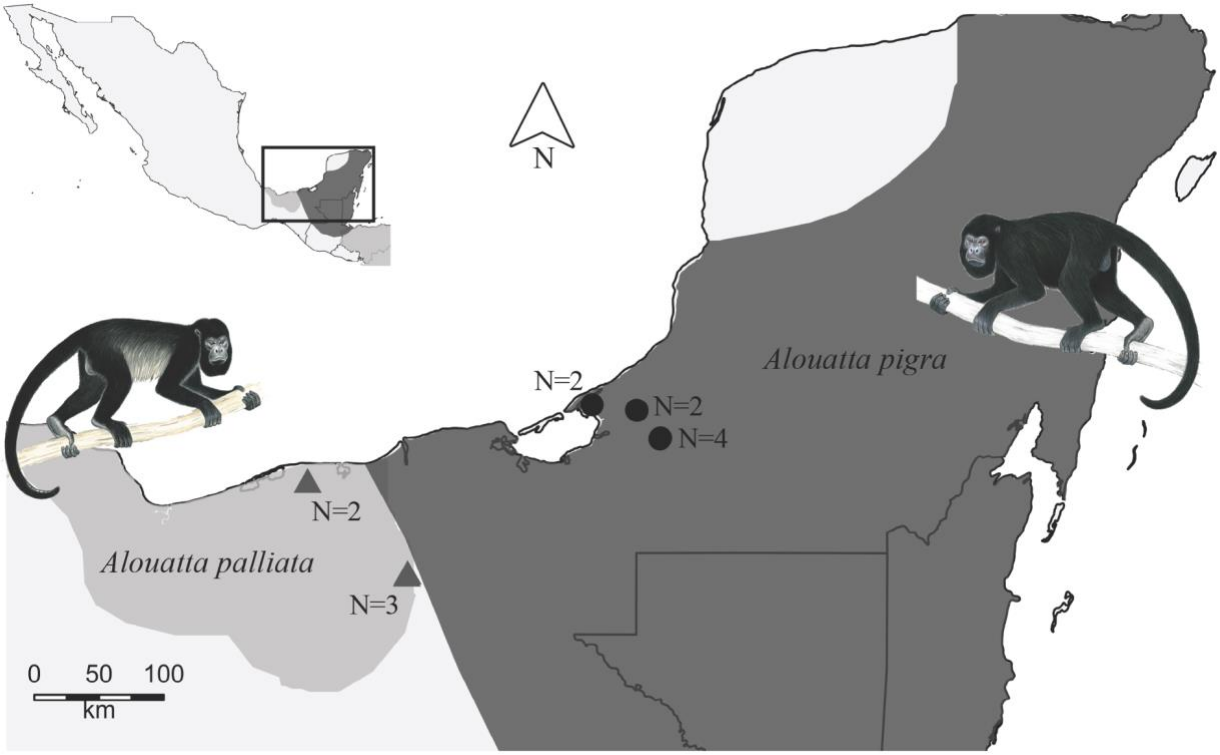


Figure 2-1: Sampling localities of individuals analyzed in this study and distribution ranges of each species.

Ranges are color-coded with *Alouatta palliata* in light gray and *A. pigra* in dark gray, according to the IUCN Red List, https://www.iucnredlist.org/. Triangles represent sampling localities for *A. palliata* individuals (N = 5); circles represent sampling localities of *A. pigra* individuals (N = 8). Drawings by Stephen Nash.

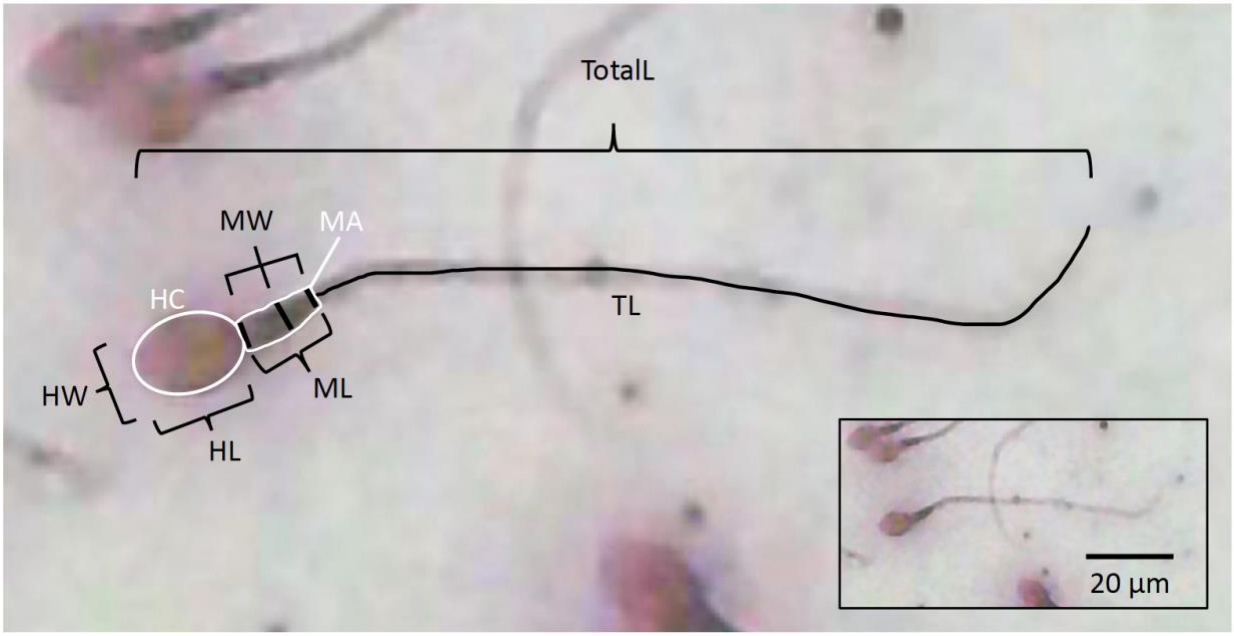


Figure 2-2: Diagram of measurements taken on sperm cells.

Sperm cells were stained with Eosin-nigrosin, and images were taken at 200X magnification with a Celestron Digital 5MP microscope camera. The embedded box in the bottom right corner shows the sperm cell without annotation and with a scale bar.
Abbreviations: HC, head circumference; HL, head length; HW, head width; MA, midpiece area; ML, midpiece length; MW, midpiece width (three measurements); TL, tail length; TotalL, total sperm length.

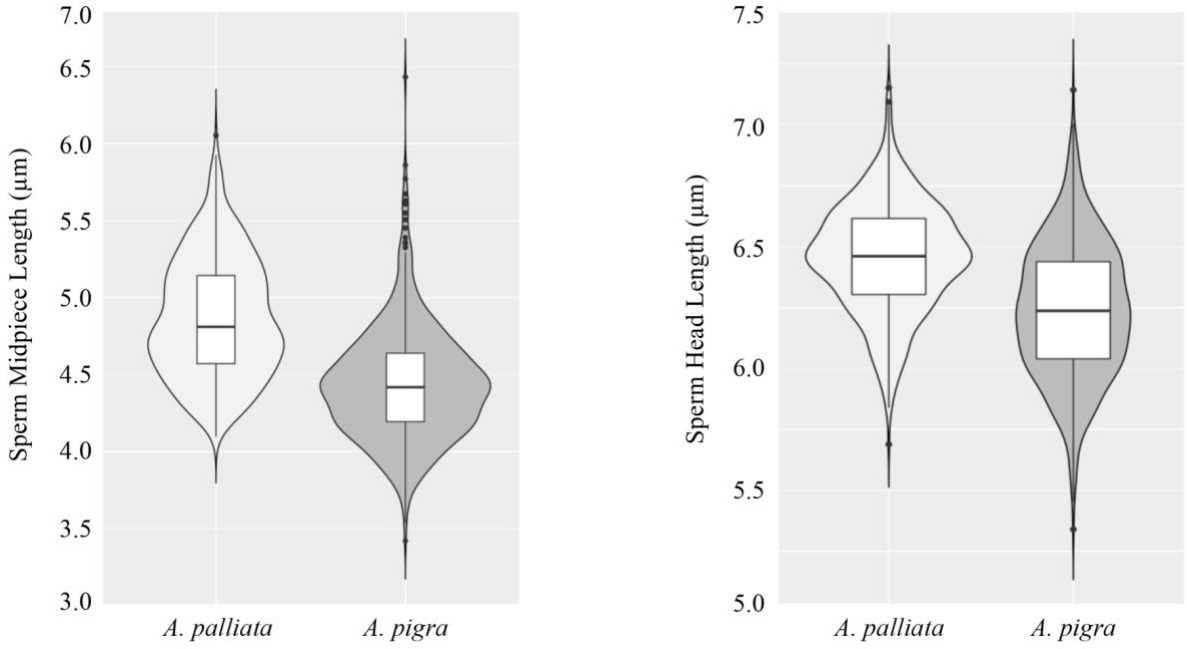


Figure 2-3: Violin plots of sperm head length and midpiece length between species.

Violin plots of sperm head length (left) and midpiece length (right) in *Alouatta palliata* (N = 500 sperm, 5 individuals, light gray) and *A. pigra* (N = 613 sperm, 8 individuals, dark gray). The boxplots show mean and standard deviation. Statistical differences between species were determined using linear mixed models in which the sperm measurement was set as the outcome variable, species was set as fixed effects, and individual was set as a random effect. The results of my models show that species identity was a statistically significant predictor of both sperm head length and midpiece length (see Table 2-2).

Chapter 3 : Directional Bias in the Occurrence of Sperm Morphological and Testicular Abnormalities in Highly Backcrossed Howler Monkey Hybrids

3.1 Abstract

Disruptions in male reproductive traits, including sperm morphology and testicular development and size, are some of the first reproductive barriers to arise during speciation. This may result from the rapid evolution of genes underlying these traits, which is driven in part by sexual selection. Interspecific differences in male reproductive traits are exacerbated when the species differ in mating system and thus in the intensity of sexual selection on an individual's phenotypes. I previously identified longer sperm heads and midpieces in *Alouatta palliata* compared to *A. pigra*, which are howler monkey sister species characterized by different mating systems, where *A. palliata* males are presumed to be under more intense sexual selection than *A. pigra* males. These species form a natural hybrid zone in southern Mexico, providing a unique opportunity to identify adaptive traits that may be selected for or maladaptive traits that may contribute to reproductive isolation in species where reproductive isolation is partially complete. I hypothesized that longer sperm heads and midpieces observed in *A. palliata* are adaptive traits positively associated with sperm competitiveness. As such, I expected that these traits would provide a fitness advantage and thus be present in all hybrids. Additionally, some of the first traits affected by hybridization involve reproduction due to hybrid incompatibilities. Thus, I expected to find more sperm and testicular abnormalities in hybrids compared to non-hybrid individuals. I used linear mixed models to determine the effect of admixture on sperm head and midpiece lengths in hybrids and found that *A. palliata*-like hybrid sperm head length mirrors the

A. palliata condition; however, *A. palliata*-like sperm midpiece length resembles the shorter midpieces observed in *A. pigra* and *A. pigra*-like hybrids. I also found that *A. palliata*-like hybrids have more abnormal sperm and testes compared to all other individuals. My results suggest that the sperm traits I measured may not provide a fitness advantage to hybrid males or that the gene complexes associated with these traits may be disrupted when the mixing of the two genomes occurs in hybrids. Further, the abnormalities in sperm and testicular traits are biased toward *A. palliata*-like hybrids, suggesting that hybridization between *A. palliata* and *A. pigra* has a stronger detrimental effect on individual males that are the product of backcrossing with *A. palliata* (i.e., *A. palliata*-like hybrids) than those produced when hybrids backcross with *A. pigra*. Further analyses will be necessary to identify the genomic basis of these differences.

3.2 Introduction

Hybridization is important in shaping the evolution of primates (reviewed in Zinner et al., 2011; Tung & Barreiro, 2017; Ackermann et al., 2019). The study of hybrid zones is a critical way to understand reproductive isolation and adaptation because the recombined genomes of backcrossed hybrids are ideal for evaluating the effects of selection on traits (Barton & Hewitt, 1985; Taylor & Larson, 2019). Hybrid zones are geographic regions where species interbreed and produce at least some viable offspring (Barton & Hewitt, 1985) and which provide unique opportunities to study traits in real time that can act as reproductive barriers or facilitate gene flow (Barton & Hewitt, 1985; Harrison, 1990; Harrison & Larson, 2014; Taylor & Larson, 2019). Certain traits can act as barriers to reproduction, or they can be adaptive, or have no effect in hybrids (i.e., act neutrally). Because hybrid incompatibilities often target reproductive traits, these are some of the first traits to diverge between incipient species due to selection against hybrids (e.g., Turner, Schwahn, Harr, 2012; Schwahn et al., 2018; Orr & Turelli, 2001). There is

rapid diversification of sexual traits, driven by sexual selection and sexual conflict (e.g., Swanson & Vacquier 2002; Panhuis et al., 2001; Maan & Seehausen, 2011; Stumpf et al., 2011). Male gametes, in particular, evolve rapidly and can differ morphologically between species and populations (e.g., Lüpold & Pitnick, 2018). Therefore, under hybridization, I would expect that some sexual traits adaptive for one species might also be adaptive for admixed individuals (e.g., Coyne & Orr, 2004; in birds Albrecht et al., 2019). Alternatively, if those traits are polygenic, or if the genes responsible for these traits interact with other parts of the genome, the new alleles acquired through hybridization might be maladaptive in hybrids (e.g., Coyne & Orr, 2004). To determine how sexually selected traits such as sperm morphology are affected by hybridization, I measured and compared sperm morphology as well as testicular abnormalities from admixed individuals within a howler monkey hybrid zone.

Interspecific variation in male reproductive traits, such as sperm morphology and testicular size, is associated with differences in the intensity of sexual selection between species. In primates, sperm morphological traits such as long sperm midpieces (Anderson & Dixson, 2002; Anderson, Nyholt, Dixson, 2005) may be advantageous to sperm competing in the female reproductive tract, where post-copulatory sexual selection via sperm competition or female cryptic choice may occur (Birkhead & Pizzari, 2002; Lüpold & Pitnick, 2018; Roldan, 2019). Differences in these sperm components may provide a reproductive fitness advantage that help an individual's sperm outcompete sperm from other males (Pitnick, Hosken, Birkhead, 2009; Fisher et al., 2016; Lüpold & Pitnick, 2018; Lüpold et al., 2020). Across species, larger testes indicate more sperm producing tissues and a larger number of sperm produced per ejaculate (e.g., in primates: Short, 1979; Möller, 1988; Harcourt, Purvis, Liles, 1995; Harcourt et al., 1981). Thus, the average testes size is another trait used to infer the intensity of sexual selection

in primates (e.g., Harcourt et al., 1981; Harcourt, Purvis, Liles, 1995; Kelaita et al., 2011). Further, the relative testes to body size ratio, or gonadosomatic index, is often used to compare across taxa and is positively correlated with the number of mates that females could have during receptive periods (Harcourt, Purvis, Liles, 1995). These observations suggests that larger numbers of sperm may be advantageous during sperm competition, such that males with more sperm and with sperm traits that successfully outcompete sperm from other males have a higher probability of fertilization (Parker, 1993; Lüpold & Fitzpatrick, 2015) and thus greater reproductive potential (Snook, 2005; Möller & Ninny, 1998; Rudolfsen et al., 2008). Despite advances in the general understanding of the effect of sexual selection on interspecific differences of male reproductive traits in primates, there is a lack of studies that analyze how primate sperm morphology and testes size are affected in interspecific hybrids produced from parental species with significant differences in these traits (but see Kelaita et al., 2013).

Abnormalities in testicular morphology and function in spermatogenesis are common in vertebrate hybrids (e.g., Turner, Schwahn, Harr, 2012; Schwahn et al., 2018). This is usually thought to be due to genetic incompatibilities during the interaction of the two divergent genomes (i.e., Dobzhansky-Müller incompatibilities) in hybrid individuals (Turelli, Barton, Coyne, 2001; Orr & Turelli 2001, Turner & Harr, 2014). In particular, the process of mature sperm cell development, or spermatogenesis, may be disrupted in hybrids resulting in the production of abnormal sperm cells, which may be unsuccessful in reaching the egg due to the negative effects of drag and immotility (e.g., Saacke, 2008; Nikolettos et al., 1999). For example, male spadefoot toad hybrids (*Spea bombifrons* x S. multiplicata) produce sperm that are significantly underdeveloped and immotile compared to those observed in either parental species (Wünch & Pfennig, 2013). In a more extreme example, most interspecific hybrid male

flycatchers (*Ficedula hypoleuca* x *F. albicollis*) exhibit azoospermia, or the absence of sperm cells in the ejaculate (Ålund et al., 2013). Further, experiments crossing different lab mouse strains, including those derived from individuals from the natural house mouse hybrid zone in Europe (*Mus musculus musculus* x *M. m. domesticus*), have demonstrated that disrupted spermatogenesis in hybrids occurs at three distinct stages of meiosis in the testes, overall indicating that spermatogenesis is negatively affected by hybridization (Oka et al., 2010). Proper testicular development is crucial for successful spermatogenesis and for determining testicular size, a measure of the amount of sperm that can be produced (e.g., Sharpe et al., 2003; Papaioannou et al., 2009; Griswold, 1998). For example, disruptions in Sertoli cell proliferation during development lead to small, malformed testes (Sharpe et al., 2003; Papaioannou et al., 2009; Griswold, 1998) and in hybrids there is widespread evidence of underdeveloped testes and early meiotic arrest (e.g., in mice: Oka et al., 2010; Wildmayer et al, 2020; in fish: Yoshikawa et al., 2018; in insects: Engels & Preston, 1979; Joly et al., 1997; Kanippayoor et al., 2020; Liang, Hodge, & Sharakhov, 2021).

Here, I analyze and compare sperm morphology and testes size and shape in a primate hybrid zone to understand how hybridization between species with significant differences in sperm and testicular morphology affects individuals with admixed genetic backgrounds (i.e., hybrids). I examined sperm morphology and testicular abnormalities from unadmixed and admixed individuals within and outside the Mexican mantled howler monkey (*Alouatta palliata mexicana*- henceforth *Alouatta palliata*) and Guatemalan black howler monkey (*A. pigra*) hybrid zone in southern Mexico (Cortés-Ortiz et al., 2007). These species diverged approximately 3 million years ago (Cortés-Ortiz et al., 2003), but now they meet in a narrow (~20 km) secondary contact zone in Tabasco, Mexico (**Figure 3-1**). This hybrid zone is well-characterized, with

genetic analyses revealing that reproductive isolation is strong (Cortés-Ortiz et al., 2007; Cortés-Ortiz et al., 2019; Baiz et al., 2019).

With regards to sperm and testicular traits, my previous work showed that *A. palliata* males have significantly longer sperm heads and midpieces compared to *A. pigra* (Hirst et al., 2023). Further, the gonadosomatic index is larger in *A. palliata* compared to *A. pigra* (Kelaita et al., 2011). Previous analysis shows that the multigenerational backcrossed hybrids in this system have relative testes volumes that mirror the species with which they share the most of their genomic background, while intermediate hybrids have more variable testes sizes (Kelaita et al., 2013). These sperm and testicular traits are thought to have evolved in response to distinctive sexual selection pressures during the evolution of these lineages due to differences in social and mating systems (Kelaita et al., 2011; Hirst et al., 2023). Specifically, in the polygynandrous *A. palliata*, females have more opportunities to mate with multiple males per receptive period (Wang & Milton, 2003; Ryan et al., 2018), and post-copulatory sexual selection is hypothesized to be more intense than in the mostly polygynous *A. pigra*. Because of the marked interspecific differences in mating systems, sperm morphology, and relative testes size between these species, I evaluated the morphology of hybrid male sperm and testes and tested whether hybrids exhibited sperm and testicular morphological phenotypes consistent with one species, the other species, or neither. I predicted that *A. palliata* sperm morphology and testicular size would be adaptive and thus introgress into admixed individuals. Due to the known extent of reproductive isolation in this hybrid system (Baiz et al. 2019; 2020), I also predicted that hybrids would have more sperm and testicular abnormalities due to errors during development, as a result of Dobzhansky-Müller incompatibilities (e.g., Orr & Turelli, 2001; Turner & Harr, 2014).

3.3 Materials and Methods

3.3.1 Ethical note and data availability

My research complied with the ethical standards set by the University of Michigan Institute of Animal Care and Use Committee (PRO00009558) and legal requirements in Mexico (SGPA/DGVS/03676/07, SGPA/DGVS/04949/07, SGPA/DGVS/03293/10, SGPA/DGVS/4936/19) and the United States (USFWS 08US178005/9, 12US46480A/9, 21US77243D/9). All data generated from this study are available either in the supplemental material or upon reasonable request to the senior author.

3.3.2 Sample collection

From 2007 to 2019, our field team conducted five sampling expeditions in Tabasco and Campeche, Mexico, in which we collected blood and ejaculate from 29 wild adult male howler monkeys (**Figure 3-1, Table 3-1**). We used a remote delivering system (Telinject USA, Inc.) to anesthetize the monkeys. We determined adulthood based on dental development and wear, considering individuals with a fully erupted dental formula and some wear to be adults. Semen samples were obtained by electroejaculation for subsequent analysis (Flores-Herrera et al., 2012; Hirst et al., 2023).

The protocol I used to collect semen samples is fully described in Hirst et al. (2023). Briefly, I inserted a lubricated electroejaculator probe in the male's rectum and delivered a series of short, low-voltage (2 - 10 V) pulses of electrical current to the prostate until the monkey ejaculated or until I completed three series. If individuals did not ejaculate after three series, I did not further attempt ejaculate collection to avoid extreme stress in the animal. I collected semen samples in sterile tubes and visually assessed the sample for color and approximate volume in

the field using a compound microscope (Swift F-31, Swift Instruments, International S. A.; or a phase-contrast CxL Binocular Cordless, LaboMed Inc., Los Angeles, CA). I diluted semen samples with sperm washing medium and mixed part of the sample with Eosin-nigrosin stain (EN), which I then spread on microscope slides and let air dry before mounting them with Entellan® mounting media. I captured images of sperm using a Celestron Digital 5MP Microscope Imager for sperm morphological analysis and abnormality quantification. After sampling, and once individuals were fully recovered from anesthesia, they were released back to the same locations where they were captured.

3.3.3 Hybrid index

Previously, the genetic hybrid index was calculated for 21 individuals used in this study that were sampled in the *A. palliata* x *A. pigra* hybrid zone in Mexico. This hybrid index was calculated using a panel of 29 microsatellite loci (Cortés-Ortiz et al., 2019) and the software *introgress* (Gompert & Buerkle, 2009; 2010). One individual did not have enough microsatellite data to include in the analysis, so I relied on the previously generated ddRAD sequencing data that was analyzed using *bgc* (Gompert & Buerkle, 2012) to generate a hybrid index (Baiz et al., 2019). It was previously demonstrated that the hybrid index estimates of microsatellite and ddRAD loci are correlated (Baiz & Cortés-Ortiz, unpublished), so for this individual I used the hybrid index based on the ddRAD estimate. The hybrid index estimate uses allelic information of individuals from the parental species outside the hybrid zone (39 *A. palliata* and 109 *A. pigra* in this case for microsatellites, Cortés-Ortiz et al., 2019 and 38 *A. palliata* and 44 *A. pigra* for ddRAD, Baiz et al., 2019) to estimate the degree of admixture of individuals in the hybrid zone. The hybrid index is a continuous scale between 0 and 1, in which 0 represents genetically unadmixed (i.e., unadmixed) individuals of one species (in this case *A. palliata*) and 1 represents

unadmixed individuals of the other species (*A. pigra*). A hybrid index of ~0.5 would be expected in an individual that is a first-generation hybrid (e.g., sired by an unadmixed *A. palliata* female and an unadmixed *A. pigra* male, or vice versa). Individuals that backcross to one or the other species or that are sired by hybrid individuals would have hybrid indexes across the scale. Here, I considered the seven individuals sampled outside the hybrid zone to be unadmixed based on their geographic distance from the hybrid zone (**Figure 3-1**).

For the statistical analyses described below, I classified individuals into four group categories based on their genetic hybrid index or geographic location. Specifically, individuals within the hybrid zone with a hybrid index of 0 or 1 were classified as unadmixed *A. palliata* and *A. pigra*, respectively, and analyzed together with individuals outside the hybrid zone. I classified individuals with a hybrid index between 0 and 0.49 as *A. palliata*-like hybrids and those with a hybrid index of 0.51 to 1 as *A. pigra*-like hybrids. Notably, the hybrid index for individuals used in this study ranged from 0 to 0.03 and 0.84 to 1. There were no recent generation hybrid males included in this study because recent generation hybrid males are rare (Cortés-Ortiz et al., 2019) and because it is impossible to identify the extent of admixture based only on phenotype (Kelaita et al., 2013), so the field team samples blindly with respect to genotype.

3.3.4 Sperm morphology data collection and reliability

I used ImageJ© (Schneider et al., 2012) to measure 100 normal sperm cells per individual when possible (*A. palliata*-like individuals: N = 8, range 35 - 100, median 100, mean 92 ± 23; *A. pigra*-like individuals: N = 8, range 10 - 100, median 100, mean 74 ± 40; base statistics for unadmixed individuals are in Hirst et al., 2023; **Table 3-2**). I considered sperm to be normal if they had an intact head, midpiece, and tail, and showed no obvious deformities in any of these

parts, as illustrated in **Figure 3-2**. I measured only the sperm head length and midpiece length (**Table 3-2**), as these were traits that differ significantly between *A. palliata* and *A. pigra* in my previous analysis (Hirst et al., 2023). To ensure that my measurements were reliable, I took three steps. First, I delegated the measuring to one person (MAH). Second, during each measuring session, I measured a single sperm and compared those measurements to previous measurements of that sperm using t-tests to ensure there was no statistically significant difference between measurements over time. Finally, I measured 20 random sperm from three randomly chosen individuals twice and compared the repeated measures using t-tests. The reliability measures for these traits showed that my sperm measurements were consistent and reliable ($p < 0.05$). Although I only measured sperm head length and midpiece length here, I recognize that sperm tail length and total length may be important. However, my unreliable tail measurements (as reported in Hirst et al., 2023) suggest that more advanced sampling and sperm smear preparation methods are required to address this issue.

3.3.5 Statistical analysis of sperm morphology

To account for the individual variation in sperm morphology, I used linear mixed models to analyze the sperm morphology data (sperm head and midpiece lengths) and determine if hybrid sperm morphology is more similar to one parental species than to the other, or if hybrid sperm are different compared to both parental species. I analyzed all raw data using the *lmer4* package (Bates et al., 2015) in RStudio (v.2022.02.0). I ran several variations of each model and used the corrected Akaike Information Criterion (AICc) to determine the models with the best fit. I chose to present four models here (referred to as models A, B, C, and D) to evaluate the differences when using categorical groups (A and B) versus the continuous hybrid index (C and D). Models A and B are the simplest and have *sperm measurement* as the dependent variable, *group category*

as the fixed effect and *individual* as random effects. Model C is the same, except that group category was replaced with hybrid index. Lastly, I implemented model D to test for a quadratic effect of the hybrid index on sperm morphology, because the hybrid index shows a bimodal distribution (Cortés-Ortiz et al., 2019). I calculated the odds ratios for each model to interpret the results of the linear mixed models. I also created fixed effect coefficient plots to visualize the effects using the *sjstats* package (v. 0.18.2, Lüdecke, 2018) in RStudio.

3.3.6 Abnormal sperm morphology

I quantified sperm abnormalities from admixed and unadmixed individuals to determine if sperm from the howler monkey hybrids have a higher prevalence of abnormalities, which has been observed in hybrid males from other vertebrates (e.g., Campbell & Nachman, 2014; Wünsch & Pfennig, 2013; Albrecht et al., 2019; Albrechtová et al., 2012; Cramer et al., 2021). I categorized each sperm as either normal or abnormal, and subcategorized abnormal sperm by the type of abnormality according to the 6th edition WHO Laboratory Manual for Evaluating Human Semen (World Health Organization, 2021; **Figure 3-2**). Specifically, I only categorized abnormalities in the sperm head and midpiece because it is difficult to observe the end of the sperm tail in my dataset (Hirst et al., 2023). To assess sperm abnormalities, I used a random number generator to choose a sperm smear slide image which I then visually scanned for sperm with the head and midpiece intact. I recorded the image numbers to ensure that each sperm cell was only counted once, and I counted 50 sperm cells per individual. I used 50 intact sperm cells from each individual except for one *A. pigra*-like hybrid, which only produced 38 sperm cells, for the abnormality assessment.

To account for the variation in sample size across group categories (i.e., *A. palliata*, *A. palliata-like*, *A. pigra*-like and *A. pigra*) and the partial missing data for one individual, I used

generalized linear mixed models (GLMMs) with a binomial distribution for my analysis of sperm abnormalities. I compared a suite of different models with slight variations, such as interaction terms, and used the AICc to select the best model. Specifically, the AICc indicated the best fit model with group category as fixed effect, numbers of abnormal versus normal sperm as the outcome variable, and individual identity as random effects. As with my linear mixed models, this model used *A. palliata* as the reference category. In my dataset, each row contained one sperm cell, resulting in 1438 sperm analyzed. I calculated the odds ratio for the GLMM and the confidence intervals for model interpretation.

3.3.7 Abnormal testicular morphology

During the field expeditions, the field team measured testicular size and recorded testicular abnormalities (e.g., lack of one testicle, shape differences, etc.) that were observed in the male individuals. After determining the hybrid index of individuals in the lab, I discovered that only *A. palliata*-like hybrids had testicular abnormalities. I performed summary statistics to compare the testicular volumes between *A. palliata*-like hybrids used in this study to highlight the abnormalities in size, but I also report other morphological abnormalities.

3.4 Results

3.4.1 Sperm morphology

I used linear mixed models to identify differences between sperm head length and midpiece length between unadmixed and admixed individuals to take individual variation and unequal sample size into consideration and used odds ratios to interpret statistically significant model results. Unadmixed *A. pigra* showed significantly shorter sperm head and midpiece lengths compared to unadmixed *A. palliata* (β = -0.21, 95% CI_{beta} [-0.33, -0.08]; OR = 0.81, 95% CI_{OR}

[0.71, 0.94]) (**Figure 3-3, Table 3-3**), as previously shown in Hirst et al. (2023). Admixed *A. palliata*-like hybrids have sperm head lengths that are not significantly different from those observed in unadmixed *A. palliata* (β = -0.05, 95% CI_{beta} [-0.18, 0.07]); however, their midpiece lengths are significantly shorter than those in unadmixed *A. palliata* (β = -0.27, 95% CI_{beta} [-0.47, -0.06]; OR = 0.77, 95% CI_{OR} [0.61, 0.96]) (**Figure 3-3, Table 3-3**). When comparing sperm of *A. pigra*-like hybrids and unadmixed *A. pigra*, I did not find any statistically significant differences in sperm head length (β = -0.07, 95% CI_{beta} [-0.23, 0.10]) or midpiece length (β = 0.06, 95% CI_{beta} [-0.17, 0.29]) between these groups. My comparison between admixed *A. palliata*-like and *A. pigra*-like males showed a significant difference in sperm head length (β = -0.21, 95% CI_{beta} [-0.33, -0.10]; OR = 0.81, 95% CI_{OR} [0.66, 0.88]), but no significant difference in sperm midpiece length (β = -0.09, 95% CI_{beta} [-0.28, 0.09]).

3.4.2 Abnormal sperm morphology

Out of all the sperm cells I screened from unadmixed and admixed individuals, I found that 15.5% (223/1438) were abnormal. From these, 4.48% (10/223) were from unadmixed *A. palliata* and 24.7% (55/223) from unadmixed *A. pigra*. I observed sperm defects in all individuals in this study, with the most common abnormality being an amorphous head, followed by small round head, tapered head, and bent neck (**Table 3-4**).

Among hybrids, I found that *A. palliata*-like hybrids had the highest proportion of abnormal sperm at 22.3% (89/400), with abnormalities mostly presenting as one or more of the following: tapered head, amorphous head, or bent neck (**Table 3-4**). I found that *A. pigra*-like hybrids had 17.8% (69/388) abnormal sperm, which presented as amorphous head, small round head, or bent neck (**Table 3-4**). To assess the statistical significance of these differences in abnormal sperm proportions, I used a GLMM with a binomial distribution and calculated the

odds ratios for interpretation. My model used *A. palliata* as the reference group. I found that *A. palliata*-like hybrids have 3.84 times the odds that a sperm will be abnormal compared to *A. palliata* (β = 1.35, 95% CI_{beta} [0.65, 2.09]; OR = 3.84, 95% CI_{OR} [1.91, 7.73]) (**Table 3-5**). I found that admixed *A. pigra*-like males did not differ in proportion of abnormal sperm cells compared to unadmixed *A. pigra* (β = 0.17, 95% CI_{beta} [-0.31, 0.67]). My comparison of abnormal sperm proportions between *A. palliata*-like and *A. pigra*-like hybrids suggested no statistical difference between the two groups (β = -0.07, 95% CI_{beta} [-0.65, 2.09]).

3.4.3 Abnormal testicular size and morphology

I observed abnormal testicular morphology in half (4/8) of the *A. palliata*-like hybrids (**Table 3-1**). Individual 25-04 has a hybrid index of 0.10 and had pear-shaped, asymmetrical testes, while the other three individuals had notably underdeveloped testicles. Unadmixed *A. palliata* males in this study had the largest average testicular volumes similar to the pattern reported in Kelaita et al. (2011), which was calculated from four out of five individuals because testicular volume data was unavailable for one individual (mean: 12.7 ± 2.49 cm^3, range: 9.27 - 15 cm^3, median: 13.35 cm^3; **Table 3-1**). Unadmixed *A. pigra* males (N = 8) had the smallest average testicular volumes (mean: 5.37 ± 1.43 cm^3, range: 3.39 - 7.48 cm^3, median: 5.47 cm^3). Average testicular volume in both hybrid groups is in-between the two extremes (*A. palliata*-like mean: 9.03 ± 3.44 cm^3, range: 4.88 - 15.8 cm^3, median: 7.76 cm^3; *A. pigra*-like mean: 6.71 ± 1.84 cm^3, range: 4.45 - 9.86 cm^3, median: 6.52 cm^3).

Among the unadmixed *A. palliata* and admixed *A. palliata*-like individuals, the admixed individual 52-03 had notably the smallest testicular volume (4.88 cm^3), resembling volumes observed in *A. pigra* and *A. pigra*-like hybrids. Individual 72-08 (hybrid index: 0.10) had an underdeveloped left testicle, while individual 72-03 (hybrid index: 0.03) from the same social

group had only one developed testicle. I did not observe abnormal testicles in any other individuals, including all unadmixed individuals, in this study (**Table 3-1**).

3.5 Discussion

Interspecific hybridization can potentially lower the reproductive fitness of unadmixed parental individuals. Thus, some of the first barriers to reproduction include abnormalities in reproductive traits, such as sperm and testicular development and function. Here, I compared sperm head length and midpiece length, as well as the proportions of abnormal sperm and abnormal testicles, between unadmixed howler monkeys that form a well-characterized hybrid zone in southern Mexico. I expected to find sperm head and midpiece lengths similar to those observed in *Alouatta palliata*, where those traits are assumed to be adaptive under intense sperm competition. However, I found that *A. pigra*-like sperm is indistinguishable from those in *A. pigra.* And, although the sperm head length in *A. palliata*-like hybrids is the same as those observed in *A. palliata*, the sperm midpiece in *A. palliata*-like hybrids resembles those observed in *A. pigra* and *A. pigra*-like hybrids. I also expected to observe more sperm abnormalities in hybrids because reproductive isolation and selection against hybrids has been observed in this hybrid zone (Cortés-Ortiz et al., 2019; Baiz et al., 2019; 2020). However, I found that all groups except unadmixed *A. palliata* have high proportions of abnormal sperm cells, with *A. palliata*-like hybrids exhibiting the highest proportion (**Table 3-4**). These results suggest that sperm head length and midpiece length do not provide a fitness advantage to hybrid males in the direction I expected, or that the gene complexes associated with these traits may be subjected to negative epistasis in a hybrid genome. Additionally, there are more sperm and testicular abnormalities in *A. palliata*-like hybrids compared to the other three group categories, suggesting that hybridization between *A. palliata* and *A. pigra* is more detrimental for *A. palliata*-like hybrids.

Further, the abnormalities in sperm and testicular traits are biased toward *A. palliata*-like hybrids, suggesting that hybridization between *A. palliata* and *A. pigra* has a stronger detrimental effect on individual males produced from backcrossing with *A. palliata* (i.e., *A. palliata*-like hybrids) than those produced from backcrossing with *A. pigra*.

There is evidence that sperm competition plays a large role in unadmixed *A. palliata* male trait evolution, such as a larger gonadosomatic index (Kelaita et al., 2011) and longer sperm head and midpiece (Hirst et al., 2023) compared to unadmixed *A. pigra*. I previously characterized and compared sperm morphology in unadmixed *A. palliata* and *A. pigra* that were either genetically confirmed to be unadmixed or that were far outside of the geographical range of the hybrid zone (Hirst et al., 2023). I identified longer sperm heads and midpieces in *A. palliata*, the polygynandrous species hypothesized to be under more intense sperm competition (Kelaita et al., 2011; Hirst et al., 2023). I hypothesized that longer sperm heads and midpieces observed in *A. palliata* are adaptive traits that increase the probability of fertilization when sperm from multiple males compete in the female's reproductive tract by increasing sperm swimming speed and/or endurance in the female reproductive tract (Hirst et al., 2023). In contrast, selection on those sperm traits from the polygynous *A. pigra* might be weaker, and selection is more likely to act more strongly on pre-copulatory traits that increase male-male competition for access to receptive females (see Hirst et al., 2023 and reference therein). Longer sperm are known to be positively correlated with sperm swimming speed in other taxa, such as *Tribolium* beetles (Godwin et al., 2017), birds (Losdat & Helfenstein, 2018; Lüpold et al., 2009; Støstad et al., 2018), and across several mammal species (Gu et al., 2019). As such, I expected to observe long sperm heads and midpieces in all admixed individuals, provided these traits could confer a reproductive fitness advantage to hybrid males.

Here, I found that *Alouatta palliata*-like hybrid sperm head length does not differ from that observed in unadmixed *A. palliata*; however, sperm midpiece length differs between *A. palliata*-like and unadmixed *A. palliata* and is indistinguishable from those observed in *A. pigra* and *A. pigra*-like hybrids (**Figure 3-3, Table 3-2, 3-3**). This is inconsistent with my prediction that longer head and midpieces would be selected for in hybrids and suggest that either the long sperm components I observed in *A. palliata* are not adaptive, or that other mechanisms affecting sperm morphology might be operating, such as the breakdown of gene complexes associated with those traits in *A. palliata*-like hybrids. It is also possible that *A. pigra* alleles that act on these traits are neutrally or adaptively introgressing into the *A. palliata*-like hybrids. I found no differences in sperm morphology between *A. pigra* and *A. pigra*-like hybrids, suggesting that hybridization with *A. palliata* does not affect sperm head or midpiece lengths in individuals with a mostly *A. pigra* genomic background.

When sperm competition is intense, selection favors the proper development of reproductive traits (e.g., Firman et al., 2015; Firman & Simmons, 2010a; Møller, 1988; 1989) and thus I expected that species with strong sperm competition such as *A. palliata* would have fewer sperm and testicular abnormalities. Indeed, my results support this hypothesis. Thus, I expected that *A. palliata* genes that confer the observed proper development of sperm and testes would introgress quickly in the hybrid zone as they would provide a fitness advantage to hybrids. However, when hybridization occurs, incompatibilities arising from the ensuing mixing of genomes can disrupt this pattern, resulting in high proportions of abnormal sperm and testes (e.g., Campbell & Nachman, 2014; Turner, Schwahn, Harr, 2012; Richard, Lachance, Proshold, 1975; Clark et al., 2010; Davis, Sosulski, Civetta, 2020). Thus, I also hypothesized that selection against hybrids would result in more sperm and testicular abnormalities compared to unadmixed

parental individuals. My results do not support the expectation of observing *A. palliata* traits in hybrids, but they do support the presence of more abnormal sperm and testes in hybrids. Specifically, *A. palliata*-like hybrids have the most observed abnormal sperm and testes when compared to all other group categories (**Table 3-4, 3-5**). This may suggest that *A. palliata*-like hybrids are negatively affected by interspecific hybridization because I observed significantly more abnormal sperm and testes in these individuals compared to all others included in this study.

I also recognize that several non-exclusive factors other than sperm and testicular morphology may be important indicators of how hybridization affects sperm in this hybrid zone. For example, I am blind to the genetic mechanisms resulting in these traits, such as negative epistatic interactions, genetic linkage and hitchhiking of other genes under selection, or conflicts resulting from combining genomic regions with different evolutionary histories. Additionally, other sperm traits may be more informative, such as ratios of sperm head, midpiece and tail, which are important for function and competitive ability (e.g., Gomendio & Roldan, 2008; Malo et al., 2006). Sperm number is also an important characteristic known to be affected in hybrids, where Sertoli cells and other testis-specific sperm-producing tissues are dysfunctional in hybrids, thus resulting in malformed testes and sperm cells (e.g., Shimazaki et al., 2022; Yoshiki et al., 1993; Castillo et al., 2012; Malone, Chrzanowski, Michalak, 2007; Ratomponirina, Andrianivo, Rumpler, 1982). Unfortunately, these factors cannot be measured given my current limited dataset. Thus, although I have disentangled a single aspect of how hybridization may affect sperm and testes in this hybrid system, there are many avenues that still need to be explored.

Sperm morphology is known to have a genetic basis, with contributing genes scattered mostly across autosomes and on sex chromosomes (Wooley & Beatty, 1967; Martinez et al.,

2022; Edme et al., 2019; Knief et al., 2017; Mossman et al., 2009; Morrow & Gage, 2001). These and other reproductive genes are known to evolve rapidly and show divergence even between closely related species, resulting in a diverse suite of reproductive genes highly tailored to different species yet serving similar functions (e.g., Swanson & Vacquier, 2002; but see Dapper & Wade, 2020). Interestingly, the subspecies of howler monkey analyzed in this study, the Mexican howler monkey, *A. palliata mexicana*, has extremely low genetic diversity (Zoonomia Consortium, 2020), possibly because it has gone through several bottleneck events throughout the species' evolutionary history (Melo-Carrillo et al., 2020). Because *A. palliata mexicana* has survived with such low genetic diversity over generations, it is possible that the homozygosity observed in this subspecies has been subjected to selective pressures that have, over generations, refined the biology of this subspecies, with gene complexes that cannot tolerate external variation. Thus, the introduction of reproduction-related alleles and subsequent heterozygosity from *A. pigra* might be causing the sperm and testicular abnormalities in the *A. palliata*-like hybrids. In a previous study using ddRAD sequencing data from 181 individuals sampled in sympatry in this hybrid zone and in allopatric populations of the parental species, I showed that introgression of autosomal loci is biased towards *A. pigra* ancestry, while the limited X-linked introgression predominantly derives from *A. palliata*, and there is no Y chromosome introgression (Baiz et al., 2020). Because the majority of sperm-related genes are located on autosomes in humans, and if I assume homology based on phylogenetic proximity of howler monkeys to humans, I can infer that many of the putative sperm-related genes in the hybrid zone likely have excess *A. pigra* ancestry based on the results from Baiz et al. (2020), and the interactions of these *A. pigra* alleles in a mostly *A. palliata* genomic background might be cumulatively negative. This would explain the abundance of sperm and testicular abnormalities

observed in *A. palliata*-like hybrids compared to other group categories. A closer inspection of candidate sperm-related genes is necessary to determine the extent of divergence and introgression patterns of such alleles in the hybrid zone.

3.6 Conclusion

In conclusion, I found that sperm and testicular morphology of *A. palliata*-like males are more negatively affected by hybridization as observed in the increase of sperm and testicular abnormalities compared to *A. pigra*-like hybrids. The shorter sperm midpieces and prevalence of testicular and sperm abnormalities in *A. palliata*-like hybrids compared to all other groups suggest that the *A. palliata*-like hybrids may also suffer from reduced reproductive fitness. *A. pigra*-like hybrid sperm are morphologically indistinguishable from unadmixed *A. pigra*, which may reflect the more relaxed sperm competition in this species or that introgression of *A. palliata* alleles do not negatively affect hybrids with a mostly *A. pigra* genomic background. Future exploration of the genes important for sperm development and function will reveal how sequence divergence between these species plays a role in the different sperm morphologies and the proportions of sperm and testicular abnormalities that I observed in this study.

Table 3-1: Testicular details for unadmixed and admixed male subjects.

The hybrid categories (*A. palliata, A. pigra, A. palliata*-like, and *A. pigra*-like) were defined based on the genetic hybrid index of these individuals, which was determined using a panel of microsatellites. Note: '*' indicates that the average testicular volume was calculated based on a single testicle; NA = data not available.

Admixture category	Individual identifier	Hybrid index	Average testicular volume (cm^3)	Notable testicular deformities
A. palliata N = 5	69-02	0	9.27	None
	77-01	0	12.8	None
	77-03	0	NA	None
	88-02	0	15.0	None
	88-03	0	13.9	None
A. palliata-like N = 8	25-04	0.10	8.02	Pear-shaped, asymmetrical
	26-03	0.08	11.7	None
	49-03	0.09	6.89	None
	52-01	0.09	15.8	None
	52-03	0.03	4.88	Very small
	72-03	0.03	7.49*	Only one testicle developed
	72-05	0.08	10.2	None
	72-08	0.10	7.24	Left testicle underdeveloped
A. pigra-like N = 8	21-3	0.89	4.45	None
	23-03	0.93	5.51	None
	47-02	0.92	7.92	None
	48-01	0.84	4.78	None
	50-01	0.94	9.86	None
	68-05	0.92	6.3	None
	73-02	0.95	6.74	None
	76-01	0.86	8.12	None
A. pigra N = 8	27-1	1	6.23	None
	29-3	1	6.60	None
	30-1	1	6.08	None
	30-3	1	4.41	None
	31-1	1	7.48	None
	32-1	1	3.94	None
	33-01	1	4.85	None
	67-02	1	3.39	None

Table 3-2: Sperm morphology measurements and details for admixed individuals.

This table provides the numbers of normal sperm measured and the mean measurements of normal sperm for each admixed individual, with means calculated based on individual sperm count. Corresponding hybrid index values can be referenced in Table 3-1. Individual identification numbers were assigned during field captures. Sperm measurements for unadmixed individuals can be found in Hirst et al., 2023.

Hybrid category	Individual	Number of sperm measured	Mean head length (µm)	Mean midpiece length (µm)
A. palliata-like	25-04	100	6.25	5.58
	26-03	100	6.09	4.41
	49-03	100	6.33	4.80
	52-01	100	6.98	4.51
	52-03	100	6.71	4.19
	72-03	100	6.40	5.15
	72-05	35	6.40	4.90
	72-08	100	6.46	4.77
A. pigra-like	21-3	10	5.38	4.17
	23-03	100	5.80	4.68
	47-02	100	5.97	5.35
	48-01	66	6.21	4.45
	50-01	100	4.44	4.44
	68-05	13	5.49	3.73
	73-02	100	5.23	4.37
	76-01	100	4.96	4.54

Table 3-3: Analyzing variance in sperm morphology across group categories and hybrid index.

This table presents odds ratio outcomes from four linear mixed models (LMMs) assessing variance in sperm head and midpiece lengths, considering four group categories: *Alouatta palliata*, *A. pigra*, *A. palliata*-like, and *A. pigra*-like (models A and B), and genetic hybrid index (models C and D). *Alouatta palliata* is the reference category. Both group category and hybrid index are used as fixed effects in models, with individual identification as a random effect to adjust for inherent individual variation in sperm morphology. Model choices were guided by minimizing Akaike's Information Criterion (AICc) corrected for small sample sizes. Models A and B corroborate longer sperm head and midpiece lengths in *A. palliata* relative to *A. pigra* (Hirst et al., 2023), while models C and D denote a significant negative correlation between hybrid index and sperm lengths. Significance codes: ***0.001, **0.01, *0.05.

Model	Predictor	Estimate (log odds)	Standard error	z value	*p* value	Odds ratio	Lower CI (OR)	Upper CI (OR)
A = lmer(HeadLength ~ Group_category + (1|Individual_identity)								
	(Intercept)	2.20	0.05	41.6	***$1.1e^{-21}$	9.06	8.11	10.1
	A. palliata-like	-0.05	0.07	-0.78	0.44	0.95	0.82	1.09
	A. pigra	-0.21	0.07	-3.06	**$5.9e^{-3}$	0.81	0.71	0.94
	A. pigra-like	-0.27	0.08	-3.52	**$1.8e^{-3}$	0.76	0.65	0.89
B = lmer(MidpieceLength ~ Group_category + (1|Individual_identity)								
	(Intercept)	1.86	0.08	22.0	***$3.6e^{-16}$	6.46	5.41	7.70
	A. palliata-like	-0.27	0.11	-2.47	**0.02	0.77	0.61	0.96
	A. pigra	-0.42	0.11	-3.90	***$7.9e^{-4}$	0.66	0.52	0.82
	A. pigra-like	-0.36	0.12	-2.96	**$7.1e^{-3}$	0.70	0.54	0.90
C = lmer(HeadLength ~ Hybrid.index + (1|Individual_identity)								
	(Intercept)	2.18	0.03	64.3	***$4.7e^{-27}$	8.85	8.25	9.49
	Hybrid.index	-0.21	0.05	-4.27	***$2.8e^{-4}$	0.81	0.73	0.90
D = lmer(MidpieceLength ~ Hybrid.index + (1|Individual_identity)								
	(Intercept)	1.72	0.06	29.0	***$5.1e^{-20}$	5.57	4.93	6.29
	Hybrid.index	-0.28	0.09	-3.04	**$5.6e^{-3}$	0.77	0.64	0.92

Table 3-4: Classification and statistics of sperm abnormalities per group category.

This table details the descriptive categories of sperm abnormalities as defined by the WHO Laboratory Manual for Human Semen Examination (World Health Organization, 2021), along with the respective quantities and basic statistics for each type of abnormal sperm within each group category. My analysis considers only sperm head and midpiece due to challenges in identifying sperm tail ends within my dataset.

Sperm Abnormality		***A. palliata*** (N=5; N_{sperm}=250)	***A. pigra*** (N=8; N_{sperm}=400)	***A. palliata*-like** (N=8; N_{sperm}=400)	***A. pigra*-like** (N=8; N_{sperm}=388)	**Total** (N=29; N_{sperm}=1438)
Head	tapered	2	8	27	4	41
	pyriform	0	6	2	2	10
	small round	3	8	14	18	43
	amorphous	1	13	13	19	46
	double	0	1	4	0	5
Midpiece	thick insertion	2	10	4	8	24
	bent neck	2	3	20	16	41
	asymmetrical	0	0	3	0	3
	thin neck	0	6	2	2	10
	Total number of abnormal sperm	10	55	89	69	223
	Proportion of abnormal sperm per group (%)	4.00	13.8	22.3	17.8	
	Proportion of abnormal sperm per 1438 total sperm (%)					15.5

Table 3-5: Analysis of sperm abnormality across group categories.

This table presents odds ratio results from a generalized linear mixed model (GLMM) using a binomial distribution, evaluating sperm abnormality variations across four group categories: *Alouatta palliata, A. pigra*, *A. palliata*-like, and *A. pigra*-like, with *A. palliata* serving as the reference category. The model contrasts abnormal and normal sperm counts, incorporating group category as a fixed effect and individual identification as a random effect, thereby adjusting for inherent individual variation. Model selection was guided by minimizing Akaike's Information Criterion, corrected for small sample sizes (AICc).

Predictor	**Estimate (log odds)**	**Standard error**	**z value**	***p* value**	**Odds ratio**	**Lower CI (OR)**	**Upper CI (OR)**
(Intercept)	-2.79	0.31	-8.99	2.4E-19	0.06	0.03	0.11
A. palliata-like	1.35	0.36	3.78	0.0001	3.84	1.91	7.73
A. pigra	1.10	0.36	3.06	0.002	3.01	1.49	6.10
A. pigra-like	1.28	0.36	3.58	0.0003	3.60	1.78	7.26

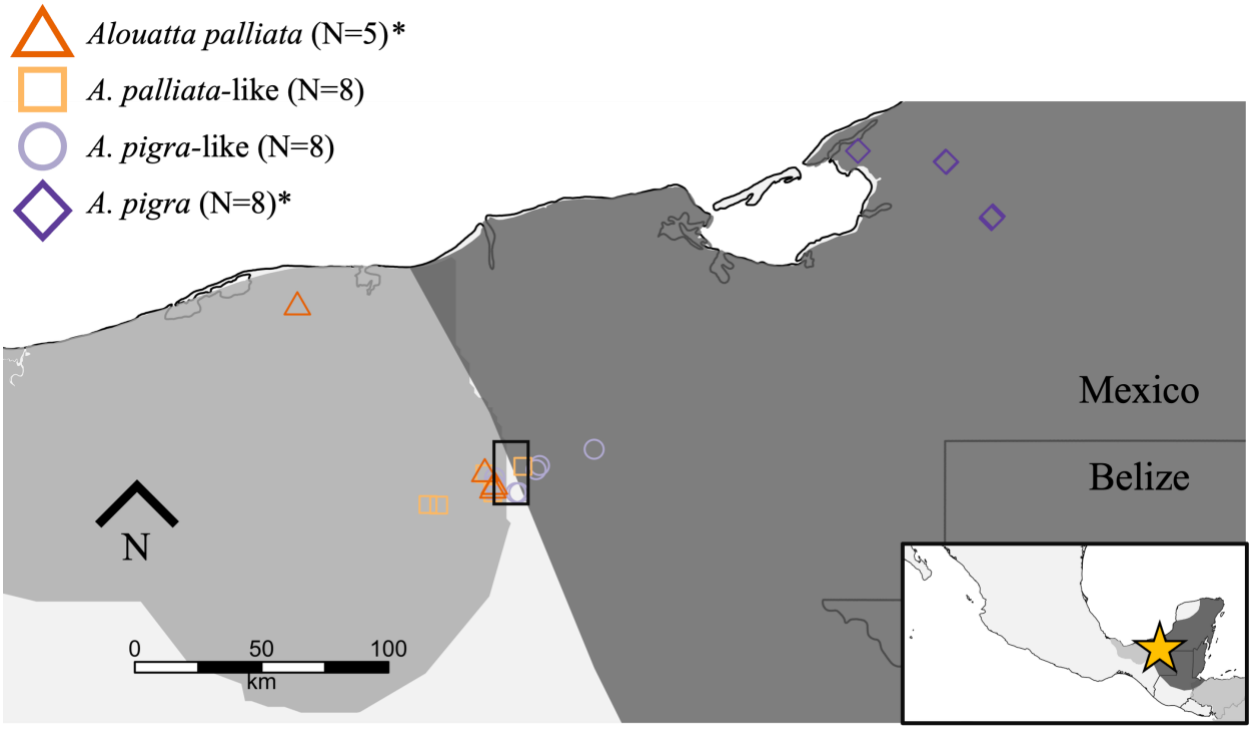


Figure 3-1: Ranges and sampling sites of *Alouatta palliata*, *A. pigra*, and the hybrid zone in Southern Mexico.

The light and dark gray areas indicate the distribution of *A. palliata* and *A. pigra,* respectively with the hybrid zone marked by a black rectangle. Sample localities are illustrated with color-coded shapes. The inset map (bottom right) provides a broader geographical context, highlighting the detailed region with a gold star. Asterisks denote samples also analyzed in Hirst et al., 2023.

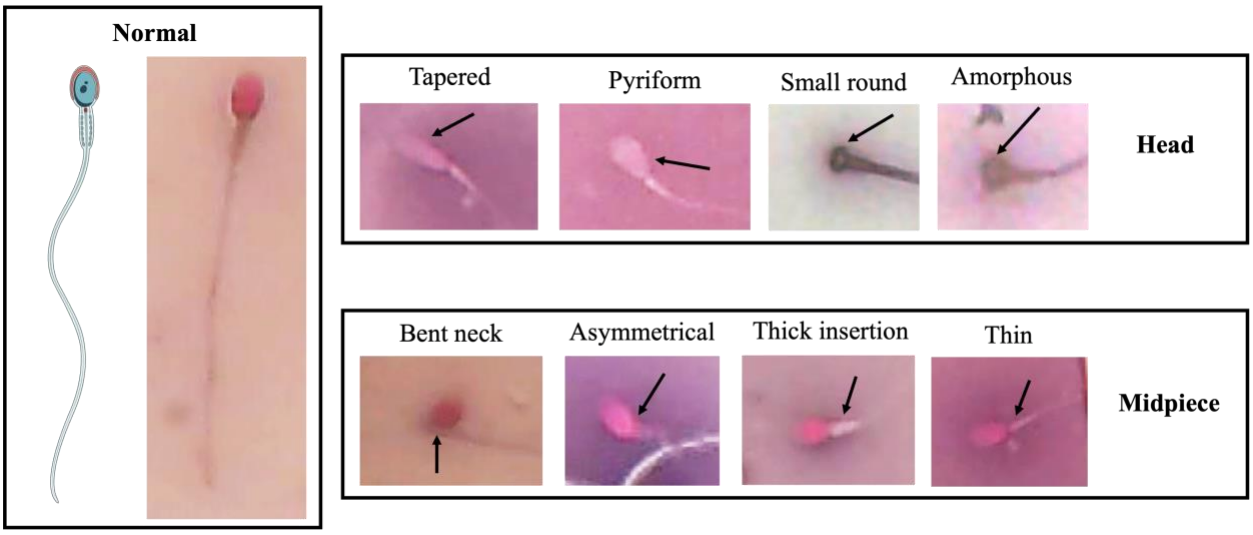


Figure 3-2: Sperm morphological abnormalities in howler monkeys.

Illustrations and images of normal and abnormal sperm from *Alouatta palliata* x *A. pigra* hybrids. The left panel shows a standard sperm drawing by John Megahan, modeled on howler monkey sperm images, juxtaposed with an image of a typical howler monkey sperm cell. The right panels reveal abnormal sperm from admixed individuals, focusing on the sperm head (top) and midpiece (bottom). Although images are not scaled equivalently and have been edited for enhanced clarity, abnormalities adhere to definitions from the 6th edition WHO Laboratory Manual for Evaluating Human Semen (World Health Organization, 2021).

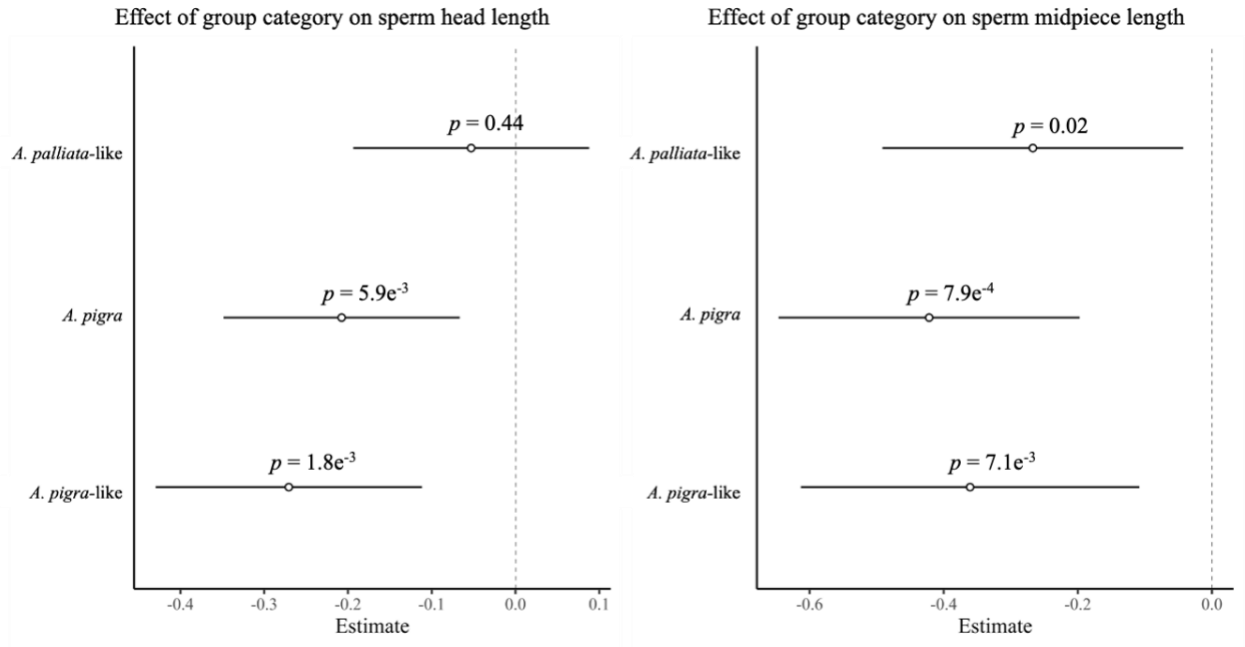


Figure 3-3: Effect of hybridization on sperm head and midpiece lengths.

Plots display fixed effect coefficient estimates from two linear mixed models (A and B from Table 3-3, respectively) exploring the influence of hybridization (via group category) on sperm head and midpiece lengths. *Alouatta palliata* serves as the reference group. Open circles represent model coefficient estimates; error bars denote confidence intervals. Effects are deemed significant when error bars do not cross the vertical zero line, with the effect magnitude and direction indicated by distance and side relative to zero, respectively.

Chapter 4 : Purifying Selection and Complex Patterns of Introgression of Putative Sperm-Related Genes in a Howler Monkey Hybrid Zone

4.1 Abstract

The rapid development of technological and analytical approaches to generate genome assemblies for non-model organisms allows us to compare genomes of closely related species to investigate genetic mechanisms arising during and after species diverge. Here I analyzed divergence of putative sperm-related genes between *Alouatta palliata* and *A. pigra*, sister species with different mating systems that form a hybrid zone. I previously found interspecific sperm morphological differences between these species, consistent with hypothesized differences in sperm competition intensity, as well as evidence of sperm and testicle anomalies in hybrid individuals. Given that many sperm traits are heritable and affected by selection, and that reproductive isolation is strong between these species, I asked if putative sperm-related genes show patterns of introgression consistent with adaptation or reproductive isolation in the hybrid zone, and how these patterns may relate to patterns observed in other instances of primate hybridization, such as between humans and archaic hominins. To answer these questions, I selected several genes that are detrimental to sperm development or function in humans and identified differences among these putative sperm-related genes between the two *Alouatta* parental species. I recognize that these putative sperm-related genes may or may not actually contribute to the phenotypes I observed in these howler monkeys, and that this study is only beginning to explore putative sperm-related genes in this howler monkey hybrid system. I built a *de novo* genome assembly for *A. pigra* and used the publicly available genome assembly for *A.*

palliata to begin exploring putative sperm-related genes by first looking at orthologs of 126 genes known to cause sperm defects in humans. I recovered exon fragments of 116 genes in both species, from which 86 had at least one base pair difference between species. I further narrowed my focus to 21 genes that were located in scaffolds for which previously generated SNP genotypes existed for admixed and non-admixed individuals to infer patterns of introgression of these genes in the hybrid zone. I found that 17 of these genes are under purifying selection, and four are under neutral evolution. Most of the SNPs associated with genomic regions near these genes show excess *A. palliata* ancestry; however, I found approximately equal instances of SNPs showing reduced and increased introgression patterns. Importantly, I found nine SNPs located between 8 to 8000 bp away from putative exonic regions of sperm-related genes, which likely reflect the pattern of introgression of those genes in the hybrid zone. Two of those genes, CATSPER3 and TEX11, show reduced introgression in the hybrid zone and have elevated expression in human testes. When comparing the set of genes to those located in known deserts of archaic ancestry in humans, I found one gene (GSTM1) located within a desert of archaic ancestry in humans that is on a genomic scaffold with an *Alouatta* SNP that shows reduced introgression. If the putative gene follows the same pattern of introgression as the SNP, it suggests that GSTM1, or genes linked to it, may be important in reproductive isolation across primates. This study has greatly contributed to the development of genomic resources, enabling a more comprehensive examination of the importance of sperm-related genes in adaptative introgression and reproductive isolation in the howler monkey hybrid zone. To that end, further studies analyzing *Alouatta* orthologs of other known sperm-related genes in humans and other organisms are needed.

4.2 Introduction

Reproductive genes are those that code for proteins involved in reproduction and are crucial for proper development, morphology, and function of reproductive traits such as gamete development and function. In animals, reproductive genes have higher rates of evolution compared to other genes (Swanson & Vacquier, 2002; Turner & Hoekstra, 2008). The evolution of reproductive genes is hypothesized to arise from sexual selection, including same-sex competition (e.g., Dapper & Wade, 2016; Swanson & Vacquier, 2002; Wilburn & Swanson, 2016; Birkhead & Pizzari, 2002; Civetta & Ranz, 2019), and cryptic female choice (e.g., Mank, 2017; Firman, Gasparini, Manier, Pizzari, 2017; Kustra & Alonzo, 2023); as well as from sexual conflict (e.g., Eberhard, 2009; Hosken, Archer, Mank, 2019; Parker & Partridge, 1998). The rapid evolution of reproductive traits and their underlying genes is especially prominent in species where females mate with multiple males during receptive periods (e.g., Swanson & Vacquier, 2002; Harcourt, Purvis, Liles, 1995; Gomendio & Rodan, 2008). In such species, selection occurs through sperm competition, in which sperm from multiple males compete in the female's reproductive tract, and only sperm with the best competitive traits reach and fertilize an egg, thus passing the genes coding for those sperm traits down to the next generation (e.g., Parker, 1970; Swanson & Vacquier, 2002).

One approach to identify targets of sexual selection in non-model organisms is to compare reproductive traits between species with varying levels of sexual selection. For example, I previously found interspecific differences in sperm morphology between two howler monkey sister species, in which *Alouatta palliata* sperm has larger heads and midpieces than *A. pigra* sperm (Hirst et al., 2023). These species also differ in mating systems (Van Belle & Bicca-Marques, 2015 and references therein), and gonadosomatic ratios (Kelaita et al., 2011). The

larger gonadosomatic ratios and longer sperm head and midpieces in the polygynandrous species, *A. palliata*, suggest more intense sperm competition compared to the polygynous species, *A. pigra*. These species form a hybrid zone (Cortés-Ortiz et al., 2007; 2019), where the new combinations of alleles of the two species in hybrid individuals are subject to selection, and the zone of contact consists of mixed groups composed of unadmixed and admixed individuals (Cortés-Ortiz et al., 2007; 2019; Dias et al., 2013). Most admixed individuals sampled in this hybrid zone are multigenerational backcrossed hybrids, with admixture proportions highly biased towards one or the other parental species (Cortés-Ortiz et al., 2007; 2019). Previous analyses of hybrid male sperm and testicular morphology show significantly more sperm and testicular morphological abnormalities in individuals with biased *A. palliata* genomic ancestry, suggesting that interspecific differences in reproductive genes or genes associated with their expression may disrupt proper development or function and contribute to reduced fitness, perhaps through Dobzhansky-Müller hybrid incompatibilities (Hirst, Chapter 2; Turelli, Barton, Coyne, 2001). However, the genes underlying these abnormal reproductive traits in howler monkeys and their patterns of introgression in the hybrid zone, which could provide information about how putative reproductive genes contribute to adaptation and reproductive isolation in this system, remain unknown.

Results from genomic introgression analyses suggest that reproductive isolation is strong in this system where the large X effect and Haldane's Rule occur (Baiz et al., 2019; 2020; Cortés-Ortiz et al., 2019). Specifically, Haldane's Rule posits that the heterogametic sex (i.e., XY males) produced from interspecific matings will be absent, rare, or sterile (Haldane, 1922; Cowell, 2023). In this hybrid zone, there is no introgression of the Y chromosome, and some anecdotal evidence suggests that an early generation hybrid male was sterile (Cortés-Ortiz et al.,

2019), consistent with Haldane's Rule. The large X effect occurs because the heterogametic sex only has one X chromosome, so recessive alleles will be expressed, even if they result in detrimental phenotypes (e.g., Coyne & Orr, 2004; Presgraves, 2018). In particular, it is well known that the X chromosome harbors many genes important for pre- and post-copulatory mating success, so the introduction of heterospecific X-linked alleles can have detrimental effects on the reproductive success of hybrid individuals (e.g., reviewed in Cowell, 2023). In the *Alouatta palliata* x *A. pigra* hybrid zone, alleles on the X chromosome exhibit reduced or no introgression, consistent with the large X effect (Baiz et al., 2019; Cortés-Ortiz et al., 2019). Thus, X-linked alleles important for reproductive success that introgress in the hybrid zone may exhibit negative epistatic interactions with nuclear genes from the heterospecific background, resulting in the patterns of reduced or no introgression of the sex chromosomes in this system. However, many reproductive genes are also located on autosomes (e.g., Ingatieva et al., 2021; Uhlén et al., 2015), especially those related to sperm development, morphology, and function (henceforth referred as "sperm-related" genes). In the howler monkey hybrid zone, species-specific autosomal gene variants with non-neutral introgression in the hybrid zone mostly show increased introgression from *A. pigra*, suggesting that some *A. pigra* alleles are adaptive in hybrids, and other variants show patterns consistent with reproductive isolation (Baiz et al., 2019). However, it is unknown if the introgression patterns of variants are associated with sperm-related genes in this system. Further, although genomic analysis allows us to track the patterns of introgression of putative sperm-related genes in the howler monkey hybrid zone, I cannot directly link reproductive traits, such as sperm and testicular morphology. Due to logistical and ethical constraints in working with wild primates, direct genetic experiments or crosses to identify genes underlying specific sperm-related traits are not possible. Additionally,

the polygenic effects that contribute to reproductive phenotypes (e.g., Civetta & Ranz, 2019; Simmons & Moore, 2009) make it difficult to resolve associations between genes and their contributions to specific sperm traits. However, I can explore sperm-related genes known in other animals, such as humans, find their orthologs in *A. palliata* and *A. pigra*, and determine the patterns of introgression of putative sperm-related alleles to determine if they might contribute to adaptation or reproductive isolation in this system.

Although I am not able to link specific genes in this study to sperm phenotypes I had previously documented, I leveraged the phylogenetic proximity of humans to howler monkeys and the extensive genotype-phenotype data available for humans to identify putative *Alouatta* sperm-related genes through homology. Sperm-related genes are well studied in humans and are dispersed throughout the genome (e.g., Ingatieva et al., 2021; Uhlén et al., 2015). To begin the exploration into putative sperm-related genes in howler monkeys, I looked at 126 human genes that are known to be detrimental to sperm in humans when genetic mutations occur in their coding regions (Ignetieva et al., 2021). This set of genes represents those that are important for sperm-related phenotypes and thus likely show at least some conservation across primates. Thus, any interspecific difference in these genes between howler monkey species may be important to sperm differences between these species. These putative sperm-related genes may or may not have an effect on *Alouatta* sperm traits; however, exploring orthologous gene regions between humans and *Alouatta* can provide information on the modes of selection acting on these putative genes in *Alouatta.* Additionally, by leveraging genomic Single Nucleotide Polymorphism (SNP) data previously generated for many individuals within and outside the hybrid zone, I can explore how SNPs located within or near putative sperm-related genes introgress in the hybrid zone to

start understanding how these putative genes may contribute to adaptation or reproductive isolation in the hybrid zone.

Given the relatively close phylogenetic proximity between humans and howler monkeys and the importance of these genes for proper sperm development and function, I expected to find orthologs of these genes in howler monkey genomes. If orthologs of these genes contribute to the same sperm functions in non-human primates, I would expect that there is strong purifying selection to maintain the sequence and thus function of these genes in howler monkeys. However, because some reproductive genes are among the most rapidly evolving (e.g., Swanson & Vacquier, 2002; Wilburn & Swanson, 2016; but see Dapper & Wade, 2020), I may find some genes in this list that are under positive selection. Additionally, because reproductive barriers are some of the first to arise during the early stages of speciation, divergence of these genes and subsequent purifying selection to maintain specific sequence or expression patterns may prevent potentially costly hybridization from occurring (e.g., Howard et al., 2009; Palumbi, 2009; Cutter, 2023; Hart & Guerra, 2017; Rowe et al., 2019). Provided these putative sperm-related genes have diverged during the time of isolation between the two parental species, genetic exchange in the *A. palliata* x *A. pigra* hybrid zone may disrupt the coordination of the genetic networks in hybrid individuals through Dobzhansky-Müller hybrid incompatibilities, reducing fitness and acting as barrier loci that contribute to species boundaries (e.g., Orr & Turelli, 2001; Turelli, Barton, Coyne, 2001). Purifying selection purges deleterious alleles that contribute to reduced fertility, and loci that diverged in sister species during the speciation process may now be under purifying selection and should thus exhibit reduced introgression in the hybrid zone if they are deleterious in a heterospecific genomic background (Barton & Hewitt, 1985; Kim & Rieseberg, 1999; Rieseberg, Whitton, Gardner, 1999). Consistent with this idea, conserved regions in the

human genome that show evidence of stronger background purifying selection have reduced Neanderthal ancestry compared to the rest of the genome (Sankararaman et al., 2014; 2016; Harris & Nielsen, 2016). As such, I hypothesized that most of the human sperm-related genes identified by Ignatieva et al. (2021) are under purifying selection in *A. palliata* and *A. pigra*, and SNPs in or near these gene regions have neutral introgression in the hybrid zone if the function of the proteins does not importantly change between parental species. Alternatively, if some of these genes have substantially diverged between the two species such that they prevent proper function in hybrids, I expect no signature of purifying selection when comparing the sequences of both species and a lack of (or reduced) introgression in the hybrid zone.

Here I used publicly available human gene sequences and the *A. palliata* reference genome assembly, as well as a newly generated *de novo* genome assembly for *A. pigra* to determine divergence and mode of selection acting on orthologs of putative human sperm-related genes. I paired this information with previously-generated diagnostic SNP data for howler monkey individuals in the hybrid zone (Baiz et al. 2019) to infer the patterns of introgression of these genes. Lastly, I compared the list of putative sperm-related genes in howler monkeys with genomic regions known to have depleted or elevated archaic ancestry in humans to determine if there are consistent patterns introgression of these autosomal genes between humans and howler monkeys (Sankararaman et al., 2014; 2016; Baiz et al. 2020).

4.3 Materials and Methods

4.3.1 Ethical note

I completed all research described here in compliance with the University of Michigan Institutional Animal Care and Use Committee (PRO00009558) and in adherence to the legal

requirements in Mexico and the United States. All data are included in this manuscript, in the supplemental material, or are available upon reasonable request from the senior author.

4.3.2 Sampling and choice of A. pigra individual for de novo whole genome sequencing

As part of an ongoing study of *Alouatta* hybridization (Cortés-Ortiz et al., 2019), our field team collected a whole blood sample preserved in lysis buffer from an adult female *Alouatta pigra* in Campeche, Mexico in 2010. I selected this specific individual for whole genome sequencing based on three considerations. First, I opted for an adult female to maintain consistency with the published *A. palliata* genome assembly (Zoonomia Consortium, 2020), which was the only available genome assembly for *A. palliata* when I started lab work, and which was also built using a sample from a female individual. Second, given the existence of a narrow hybrid zone between *A. palliata* and *A. pigra* in southern Mexico (Cortés-Ortiz et al., 2007; Cortés-Ortiz et al., 2019), I chose this individual because it was collected far away from the hybrid zone (Baiz et al., 2019). The chosen individual for sequencing yielded 279 ng/µL DNA, with the 260/280 and 260/230 NanoDrop® ratios around 1.8 and above, respectively, signifying the high-quality of the DNA extraction including the lack of contaminants such as proteins or organic solvents.

4.3.3 DNA extractions and whole genome sequencing

I extracted genomic DNA from the whole blood sample using the QIAGEN® DNeasy® Blood & Tissue kit, following the manufacturer's protocol with the following modifications: 1) I used 100µL whole blood to 100µL Buffer ATL, 2) I extended the incubation period to overnight, 3) I eluted the DNA in 100µL dH2O twice using the same spin column to maximize DNA yield. To determine the DNA concentration, I performed three measurements using the Qubit® HS dsDNA quantification kit (Thermo Fisher Scientific, Inc.) for the Qubit® 3.0 Fluorometer

(MAN0010866), and three measurements using the NanoDrop® (Thermo Fisher Scientific, Inc.). After completing the extractions, I sent the extraction to the University of Michigan's Sequencing Core library preparation with the NEBNext® Ultra™ II FS DNA kit (New England BioLabs, Inc.) for Illumina with 5 cycles of PCR. The prepared libraries were sequenced on a shared flow cell (3.75%) using the Illumina NovaSeq 6000 platform (Illumina, Inc.) for 300 cycles, resulting in 150 bp paired-end reads. The technician at the sequencing core used Bcl2fastq2 Conversion Software (Illumina, Inc.) to convert the raw BCL files into de-multiplexed Fastq files. These Fastq files were used to build the genome assembly after conducting standard quality control measures on the raw reads, as outlined below.

4.3.4 **Alouatta pigra** *genome assembly*

To ensure the accuracy of my downstream analysis, I first subjected the raw sequencing reads to rigorous quality control. Using FastQC (Andrews, 2010), I examined the integrity of the raw sequences in the Fastq files. Subsequently, I used Trimmomatic (Bolger, Lohse, Usadel, 2014) to trim reads that had a Phred score below 33. For the leading parameter, I used a value of 3 to ensure that bases from the start of a read with a quality below 3 were removed. The trailing parameter was also set to 3, meaning that bases at the end of a read were trimmed until a base with a quality score of 3 or more was encountered. I set the illuminaclip parameter as TruSeq3-PE:2:30:10 to remove any remaining Illumina adapters, and defined a strict sliding window of 5:20, meaning that I evaluated the average quality of every 5-base window along the read, and if the average quality within that window dropped below 20, the read was trimmed at the beginning of that window. I set the minimum length to 50 bp, discarding any reads that were shorter than 50 bases after trimming. Finally, I re-assessed the quality of the reads with FastQC. Given that the sequencing was conducted on the Illumina platform, which generates short-reads, I chose to

assemble the genome using SPAdes (Bankevich et al., 2012), a specialized short-read *de novo* genome assembler. I then used QUAST (Gurevich et al., 2013) to evaluate the quality of the resulting genome assembly. To determine the depth and consistency of the assembly, I calculated its coverage using BBMap (Bushnell, 2014). I have included the specific code used throughout the pipeline in **Table 4-1**.

4.3.5 Identification of putative sperm-related genes in howler monkeys

Because it is not feasible or ethical to conduct crosses or genetic experiments with wild primates to identify the gene regions responsible for sperm development, morphology, and function, I relied on data derived from human research with the caveat that the putative genes identified in *Alouatta* may or may not affect sperm in these species. Specifically, I narrowed the focus to 126 human genes known to harbor mutations that cause spermatogenic defects in humans (Ignetieva et al., 2021). The majority of these 126 genes are autosomal in humans (113), while five are X-linked, three are Y-linked, and five are mitochondrial (Ignatieva et al., 2021). Mutations in the protein coding regions of these genes are deleterious, and individuals carrying these mutations have reduced reproductive fitness in that they suffer from low sperm concentrations, abnormal sperm morphology, reduced sperm motility, or a combination of the three (Ignatieva et al., 2021). I obtained the coding sequences (i.e., exons) of these genes from e!Ensemble BioMart (Cunningham et al., 2022) in fasta format. I then employed a BLAST® (Altschul et al., 1990) query with an 80% query coverage threshold to align the human exons to the published *A. palliata* genome assembly. I extracted all of the sequence hits from *A. palliata* (i.e., exon sequences shared between humans and *A. palliata*) and aligned all reads (i.e., sequence fragments) of the same gene to create consensus sequences for each gene exon using Sequencher (v 5.4.6). I then queried the *A. palliata* consensus sequences to the *A. pigra* genome assembly,

and extracted the matching *A. pigra* sequences (i.e., putative orthologous sequences between *A. palliata* and *A. pigra*). I aligned all the reads from the same gene and created consensus sequences per gene exon for *A. pigra*. To verify that short fragments or those with low percent identity compared to the majority of fragments were indeed representative of my target genes, I cross-referenced them with the NCBI database by querying the howler monkey sequences to the entire GenBank database. I removed sequences that failed to align with the genes of interest. I performed pairwise alignments between the full human gene sequences (downloaded from e!Ensemble BioMart), including introns and exons, and the consensus sequences of *A. palliata* and *A. pigra* exon sequences from putative genes using Sequencher with at least 15 bp overlap and 70% sequence similarity. To determine the correct reading frame for each exon of the howler monkey gene sequences, I used the full human gene alongside known reading frame information for humans on the NCBI database. After aligning the sequences from each species, I removed the human intronic regions, and concatenated each species-specific sequence into a unique sequence that I used to represent partial exonic sequences of the gene for each howler monkey species.

4.3.6 Tests of selection

I further narrowed down my list of target sperm-related genes based on the availability of SNPs in scaffolds that contained these genes to determine introgression patterns in the hybrid zone (see next section). This narrowed my focus to only 21 genes. Once I obtained gene sequences for each species that were in the same reading frame, I compared sequences from the three species to determine the mode of selection acting on each gene. I used MEGA11 (Tamura et al., 2021; Stecher et al., 2020) to set humans as the outgroup and performed pairwise comparisons using codon-based Fisher's exact test of selection (e.g., Zhang et al., 1997; 1998) because of my small sample size (Zhang et al., 1997). I estimated the numbers of nonsynonymous to synonymous

substitutions between sequence pairs using the modified Nei-Gojobori method (e.g., Nei & Kumar, 2000) with the assumption of a transition/transversion bias of 2. I also set the parameters to treat positions with less than 95% site coverage as partial deletions. The null hypothesis in this model is neutral evolution, and *p*-values less than 0.05 suggest positive selection. Purifying selection is indicated by a *p* value of 1, which occurs when the observed number of synonymous differences per synonymous site is greater than the number of nonsynonymous differences per nonsynonymous site (Nei & Kumar, 2000).

4.3.7 Patterns of introgression and links with archaic hominin hybridization

To determine the patterns of introgression of these 21 putative sperm-related genes in the hybrid zone, I leveraged a SNP dataset generated from ddRADseq from unadmixed and admixed individuals that was previously used to examine genome-wide patterns of introgression (Baiz et al., 2019) using Bayesian genomic cline analysis via *bgc* (Gompert & Buerkle, 2012). In this analysis, the patterns of introgression of these diagnostic SNPs (i.e., those that are significantly associated with one species or the other) are evaluated based on two parameters: alpha and beta. Alpha refers to the direction of introgression into one species or the other, while beta refers to the amount of introgression compared to the genome wide expectation of neutrality, where loci with increased introgression are interpreted as putatively adaptive and loci with reduced introgression suggests barriers to reproduction (Gompert & Buerkle, 2012). If a SNP is not an outlier for either parameter, it is considered to be neutral (Gompert & Buerkle, 2012). To determine if the putative sperm-related genes I analyzed contain SNP outliers, I identified the *A. palliata* genome assembly scaffolds where these putative sperm-related genes were located and determined whether any previously identified SNPs were located on those scaffolds. I then noted the location of the SNPs relative to the putative sperm-related gene exon sequences that I obtained and

looked at the patterns of introgression of those SNPs in the hybrid zone (Baiz et al., 2019). Although many SNPs were very close (e.g., within 9,000 bp) to the sequences of the focal genes, none were located inside the coding sequences themselves. However, I expect that the patterns observed in SNPs proximate to the focal genes would be associated with the introgression of these gene regions, due to the unlikely presence of recombination breakpoints.

I adopted the model used in Baiz et al. (2020) to determine if any of the genes of interest in this study are located in known deserts of archaic ancestry or regions of elevated archaic ancestry in humans as defined by Sankararaman et al., (2014; 2016). Specifically, Baiz et al. (2020) examined X-linked loci with reduced introgression in the howler monkey hybrid zone and compared them with regions of the human genome that are depleted from Neanderthal or Denisovan ancestry (known "deserts" of archaic ancestry) and found a SNP outlier associated with a desert of archaic ancestry in humans. The combined evidence of a SNP outlier showing reduced introgression in the howler monkey hybrid zone with the homologous region located in a desert of archaic ancestry in humans suggests a potentially shared genomic basis of reproductive isolation in primates (Baiz et al., 2020). However, most sperm-related genes are autosomal, thus I examined the autosomal deserts of archaic ancestry in humans to determine if any SNP outliers associated with the sperm-related genes of interest in this study are within those regions.

4.4 Results

4.4.1 de novo genome assembly

The whole-genome sequencing of the *A. pigra* sample produced 354,394,574 reads, exhibiting a GC content of 41% (**Table 4-2**). After trimming the raw reads that did not meet my filtering criteria, I retained 296,687,342 reads ranging in length from 50-151 bases and with a GC content of 40%. The genome assembly resulted in 139,436 contigs, encompassing a total of

2,461,750,957 base pairs. The overall GC content of the assembly is 40.71%, with 99.21% of the reads having been constructed in the assembly. Overall, the genome assembly has an N50 value of 26,980 with the largest scaffold consisting of 265,688 base pairs, and an average of 29.09X depth of coverage (**Table 4-2**).

4.4.2 Identification of putative Alouatta sperm-related genes

Of the 126 human genes associated with spermatogenic defects (Ignatieva et al., 2021), I excluded the three Y-linked genes from the analysis because the howler monkey genome assemblies are derived from female individuals. I also excluded the five mitochondrial genes because they are subject to different selective pressures compared to nuclear genes (Vawter & Brown, 1986) and because I was mainly interested in identifying nuclear genes. From the list of 126 genes human sperm-related genes, I successfully identified exon sequences from 106/113 autosomal genes and 5/5 X-linked genes in both *Alouatta* genome assemblies (**Table 4-3**). I did not successfully find the other seven genes in the *Alouatta* genome assemblies, perhaps due to the incompleteness of both assemblies, or because they have greatly diverged from the human sequences.

4.4.3 Analysis of selection on putative Alouatta sperm-related genes

I tested 21 putative sperm-related genes for modes of selection in MEGA11 using Fisher's exact test and found no evidence of positive selection in these gene coding regions, i.e., there were no statistical comparisons between the dN/dS ratios between *A. palliata* and *A. pigra* that showed a *p*-value of 0.05 or less (**Table 4-3**). Despite the presence of some non-synonymous substitutions between the sequences of *A. palliata* and *A. pigra*, most of the genes I explored showed evidence

of purifying selection, where $p = 1$ (N = 17 genes, **Table 4-3**). The other four genes showed evidence of neutral evolution, where $0.05 < p < 1$ (**Table 4-3**).

4.4.4 Patterns of introgression in the hybrid zone and associations with archaic ancestry in humans

I identified 52 SNPs that were identified as outliers in previous introgression analysis of this hybrid zone (Baiz et al., 2019) and were located on the same genomic scaffolds as the 21 genes of interest in my analysis (**Table 4-3**). These SNPs were located across 19 different scaffolds. Each scaffold had between 1-6 SNPs, with an average of 2.74 ± 1.4 and a median of 2 SNPs per scaffold. The majority of SNP outliers (31/52) had a negative alpha value, implying an excess *A. palliata* ancestry. Only 16 SNPs showed patterns of excess *A. pigra* ancestry. I found nearly equal instances of SNPs with a positive (20/52) or negative (23/52) beta outlier, which indicates reduced and increased introgression, respectively. Most SNPs (N = 27) showed negative alpha (i.e., excess *A. palliata*) with an equal share of negative beta (i.e., increased introgression, N = 14), or positive beta (reduced introgression, N = 13). Nine SNPs were alpha, but not beta, outliers, and five were beta, but not alpha, outliers (**Table 4-3**).

However, many SNPs were not physically close to the gene sequences of interest, i.e., they were further than 100,000 bp upstream or downstream from the exon sequences of the putative sperm-related genes (**Table 4-3**). However, nine SNPs were within 9,000 bp from an exon (range: 8 – 8,562 bp, mean: 3,477 bp, median: 2,817 bp), suggesting they are likely physically linked to these genes. These SNPs were closed to the genes AHR, AK7, ARMC2, CATSPER3, DNAH11, ETV5, EPHX2, GNAO1, and TEX11. Eight of these genes showed evidence of purifying selection, and one (DNAH11) showed neutral evolution. Of these nine SNPs, one showed excess *A. pigra* ancestry and increased introgression, following the genome-

wide pattern of the majority of SNP outliers (Baiz et al., 2019). One SNP showed excess *A. pigra* ancestry and decreased introgression, one showed excess *A. palliata* ancestry and increased introgression, four showed excess *A. palliata* ancestry and decreased introgression, one showed excess *A. palliata* ancestry but was not a beta outlier, and one showed decreased introgression but was not an alpha outlier. Notably, four out of nine of these SNPs were outliers for excess *A. palliata* ancestry and reduced introgression, which is the opposite of the genome-wide pattern of SNP outliers (Baiz et al., 2019), but the same patterns observed for X-linked SNPs outliers (Baiz et al., 2020). The four SNPs that are outliers for both excess *A. palliata* ancestry and reduced introgression are located near the AHR, DNAH11, GNAO1, and TEX11 genes. The closest SNP to a gene sequence of interest was located 8 bp away from a sequence in the CATSPER3 gene and showed excess *A. pigra* ancestry and reduced introgression.

To start exploring if these putative sperm-related genes contribute to reproductive isolation or adaptation more broadly in primates, I adapted the methods used by Baiz et al. (2020) and compared the human chromosomal locations of the 21 putative sperm-related genes to known regions in the human genome that are depleted or elevated for archaic ancestry (Sankararaman et al., 2014; 2016). In total, I found six of the putative sperm-related genes in these regions: four in regions of depleted archaic ancestry (two within, and two nearby) and three within regions of elevated archaic ancestry. There are six SNPs located across two howler monkey scaffolds containing the two genes that are within regions of depleted archaic ancestry in humans, and two SNPs on a howler monkey scaffold containing the gene that are located in a region of elevated archaic introgression in humans. Four of the six SNPs associated with human regions of reduced archaic ancestry show excess *A. palliata* ancestry and reduced introgression in howler monkeys. However, all of the SNPs in the genomic scaffolds that contain these genes

associated with depleted archaic ancestry in humans are located more than 10,000 bp away from an exonic region, reducing the probability that their patterns of introgression actually represent those of the putative sperm-related genes.

4.5 Discussion

The rapid evolution and subsequent diversification of reproductive genes, such as those important for sperm function, are prime candidates as barrier loci because reproductive traits are often the first targets of selection against hybrids during hybridization events (e.g., Howard et al., 2009; Clark et al., 2010; Servedio & Boughman, 2017; Mendelson & Safran, 2021). However, reproductive genes could also contribute to adaptive introgression if their function in the admixed genomic background provides a fitness advantage (e.g., Barton & Hewitt, 1985). In this study, I generated the first draft genome assembly for the endangered Mesoamerican black howler monkey, *A. pigra*, to compare gene sequences between this and its sister-species *A. palliata*, with which it forms a hybrid zone in Mexico where reproductive isolation is strong (Cortés-Ortiz et al., 2019; Baiz et al., 2019). Specifically, I compared sequences from this novel *A. pigra* assembly with the published *A. palliata* genome assembly (REFERENCE) to delve deeper into putative sperm-related gene sequence divergence and introgression between these sister-species. In previous studies within this dissertation, I showed differences in sperm phenotype between these species (Hirst et al., 2023; Chapter 2), as well as sperm and testicular abnormalities in admixed individuals with biased *A. palliata* genomic ancestry (Chapter 3). To identify putative sperm-related genes in these species, I used a list of 126 genes in which point mutations have been linked to sperm defects in humans (Ignatieva et al., 2021). Out of these, I identified homologs for exons in 111 genes in both the *A. palliata* and *A. pigra* genome assemblies. Twenty-one of these genes are located in scaffolds for which previous studies

identified SNPs in the hybrid population that are diagnostic for each parental species (Baiz et al. 2019; 2020). After comparing and analyzing these sequences between humans and both howler monkey species, I found that most of these genes are under purifying selection, and that most SNPs located near these genes have a strong bias towards *A. palliata* ancestry. These different patterns of introgression reflect the complexity of the role of reproductive genes in hybridization outcomes. On the one hand, some of the putative sperm-related genes with reduced introgression in this hybrid zone might be contributing to reproductive isolation between the two parental species, as it has been suggested for multiple reproductive genes in other hybrid systems (e.g., in mice: Turner & Harr, 2014). On the other hand, genes with increased introgression and increase *A. palliata* ancestry suggest that alleles in *A. palliata* might provide adaptative advantage to hybrids. Understanding the extent to which sperm-related genes are responsible for reproductive isolation or adaptation will require further identification and introgression analyses of a larger set of genes across the genome.

Although many of the SNPs I examined are located in the same scaffolds of the putative sperm-related genes, most were located tens of thousands of base pairs away from putative exons (suggesting they might not be linked with those sequences). I found nine SNPs within 8,000 bp of a putative exon, which are more likely to reflect the pattern of introgression of those genes. Two of those SNPs showed reduced introgression and are associated with CATSPER3 and TEX11, genes that show elevated expression in human testis. After comparing patterns of introgression of SNPs located near some putative sperm-related genes in regions of the human genome that are known to have depleted or elevated archaic ancestry from ancient hybridization events with Neanderthals and Denisovans, I found that many of these autosomal loci in the howler monkey hybrid zone also show excess *A. palliata* ancestry and reduced introgression,

suggesting a potential role of these genes (or gene regions) in reproductive isolation among primates. Understanding the extent to which sperm-related genes are responsible for reproductive isolation or adaptation will require further identification and introgression analyses of a larger set of genes across the genome.

4.5.1 Most putative Alouatta sperm-related genes show signatures of purifying selection

There are thousands of genes known to be important for sperm development, function, and morphology. For example, a simple query for "sperm" in The Human Protein Atlas, a database created through a large collaboration of researchers documenting human gene presence, expression, and proteomes across human organs (Uhlén et al., 2015), results in a list of nearly 2,000 genes. This figure, however, may not be exhaustive given the polygenic nature of many of these traits (e.g., Massart et al., 2011). Regardless, extensive studies indicate that most sperm-related genes, especially those under strong sexual selection, exhibit significant sequence divergence and signs of positive selection (Swanson & Vacquier, 2002; Wilburn & Swanson, 2016). Sperm-related genes might be subjected to different types of selection at different times under fluctuating environments, so evidence of selection acting on these genes needs to be interpreted cautiously (e.g., Dapper & Wade, 2020). My examination of a relatively small number of genes that are critical for sperm attributes in humans, suggest conservation of these genes in primates. Indeed, my findings underscore the significance of purifying selection acting on sperm-related genes, suggesting that extreme sequence divergence in these genes may not be possible in the howler monkeys studied here due to functional constraints for proper sperm development and function, despite ~3 million years of divergence (Cortés-Ortiz et al., 2003). It is worth noting that by focusing on coding regions, I may have overlooked other vital aspects of these genes, namely regulatory regions, which could be the target of more intense selection than

coding regions given their role in dictating transcript and protein volumes. Thus, while I observe purifying selection safeguarding gene structure and function between closely related species in this study, targets of adaptation might lie more in patterns of gene expression. Comprehensive testicular transcriptomic studies could clarify the importance of sperm gene sequence divergence versus gene expression divergence in this hybrid system (Yang et al., 2022).

While the majority of the genes I studied show evidence of purifying selection, exceptions like AURKC, DNAH11, STX2, and TSPYL1 show neutral evolution. Interestingly, the STX2 gene, which is important for nuclear development during meiosis and later acrosomal reaction (Fujiwara et al., 2013), also shows evidence of positive selection between *Alouatta* and humans ($p = 0.03$), highlighting possible evolutionary shifts related to sperm nuclear development and acrosomal reaction in the evolutionary history of primates. Further exploration of this gene as a candidate for studying the importance of sperm-egg binding interactions between these species is necessary. In this study I only focused on a small number of putative sperm-related genes for which I had genomic SNP data from parental and admixed populations. However, my new *A. pigra* genome assembly and the strategy used in this study will allow us to further explore the effect of hybridization on important reproductive genes in primates using whole genome resequencing.

4.5.2 Introgression patterns of putative sperm-related genes are complex

All of the 21 putative sperm-related genes of interest were located on scaffolds that contain between 1 - 6 SNPs, and the length of the scaffold is not correlated with the number of SNPs each scaffold contains ($r = 0.36$, $df = 19$, $p = 1.6$). The patterns of introgression of these SNPs varied both within and between scaffolds (**Table 4-3**). For example, there are only two scaffolds with multiple SNPs that, as expected, all have the same pattern of introgression (**Table 4-3**).

These scaffolds contain two SNPs near the DNAH11 gene and three near the AURKC gene. The two DNAH11-associated SNPs are located within 2,000 and 12,000 bp of putative coding regions of the gene and show patterns of excess *A. palliata* ancestry and reduced introgression in the hybrid zone. The three AURKC-associated SNPs are located between 27,000 and 132,000 bp away from putative coding regions. However, the available dataset does not allow disentangling whether the observed pattern of introgression in these SNPs are due to physical linkage and lack of recombination with this or the other genes in the scaffold, or actually reflect the pattern of introgression due to selection acting on the AURKC gene. Additionally, due to the fragmented nature of the *Alouatta* genome assemblies, I did not capture all coding regions or the complete lengths of each gene, which limits the understanding of gene diverge between these species. Nevertheless, these three AURKC-associated SNPs show patterns of excess *A. palliata* ancestry and increased introgression.

Broadly, most of the SNPs located on the same scaffolds as the genes of interest show excess *A. palliata* ancestry, which is the opposite of the pattern reported by Baiz et al. (2019) that showed most SNP outliers had *A. pigra* ancestry but aligns with the X chromosome introgression pattern of excess *A. palliata* ancestry (Baiz et al., 2020). Importantly, only one gene associated with a SNP in this study is X-linked (TEX11) and shows excess *A. palliata* ancestry and reduced introgression; the remaining genes are autosomal. My observation of SNPs associated with these genes showing mostly excess *A. palliata* ancestry provides some support to my previous hypothesis that *A. palliata* sperm traits are adaptive and quickly introgress in the hybrid zone (Hirst et al., 2023). However, the fact that I do not see a sweep of *A. palliata* sperm traits in the hybrid zone is not necessarily unexpected, due to the small number of putative sperm-related genes I explored in this analysis, which may not be associated to the particular morphological

traits I previously analyzed. The presence of excess *A. palliata* variants near these genes may also reflect linkage to genomic regions that are adaptive and have yet to be broken by recombination in hybrid individuals. As previously stated, further analysis of introgression of all other sperm-related genes is needed to clarify their role in adaptive introgression.

Of the eleven putative sperm-related genes with SNPs located within 10,000 bp from an exon, six show reduced introgression. Three of those genes (DNAH11, CATSPER3, and EPHX2) are involved in sperm motility. The CATSPER3 gene has a SNP located 8 bp away from an exon showing excess *A. pigra* ancestry and reduced introgression, and in humans functions in hyperactive sperm motility during capacitation, a change in sperm motility pattern from progressive to asymmetric flagellar beating within the female reproductive tract (e.g., Jin et al., 2007; Jin & Yang, 2017). Thus, the development of sperm motility may be an important factor contributing to reproductive isolation between these species. However, because I was unable to reliably measure the sperm tail (Hirst et al., 2023) and I was not able to analyze sperm motility in my previous studies, I do not yet know if there are interspecific differences in these traits that could support this hypothesis. Future fieldwork incorporating videography of sperm motility in hemacytometers is necessary to analyze differences in sperm motility in this system.

4.5.3 Links between gene regions with increased or reduced archaic hominin hybridization and putative sperm-related gene introgression in howler monkeys

Ancient admixture between humans and archaic hominins, such as Neanderthals, Denisovans and ghost lineages (Durvasula & Sankararaman, 2020), has shaped the modern human genome, with some contemporary humans carrying as much as 6% archaic DNA (Sankararaman et al., 2014; 2016). By mapping the regions of enriched or depleted archaic ancestry in the human genome, Sankararaman et al. (2014; 2016; Durvasula & Sankararaman, 2020) provided a means

to compare genomic regions of reduced or increased introgression between hominins in the past with patterns of introgression of contemporary primate hybrid zones to locate shared loci important for adaptation and reproductive isolation across primates. Specifically, genomic regions depleted of archaic ancestry across contemporary human populations (i.e., "deserts" of archaic ancestry) are thought to be prime candidate regions for reproductive isolation between humans and ancient hominins (Sankararaman et al., 2014; 2016). Conversely, regions with elevated archaic ancestry in human populations are likely maintained because they provide a fitness advantage to modern humans, they are linked to advantageous alleles, or because these regions are neutral and were not purged by natural selection. As such, I compared the genes in deserts (i.e., candidate barrier loci) and in regions of elevated archaic ancestry (i.e., candidate adaptive loci) in modern humans with the contemporary *A. palliata x A. pigra* hybrid zone to identify parallels in candidate barrier and adaptive loci between different systems. For example, Baiz et al. (2020) found overlap between putative X-linked genomic sequences in the howler monkey hybrid system and known deserts of archaic ancestry in humans. These findings suggest a potential common mode of reproductive isolation via the X chromosome in these two systems. Additionally, there is evidence that deserts of archaic ancestry are enriched in genes that have elevated expression in human testes (Sankararaman et al., 2014; 2016) and contain a significant number of genes that are transcribed in meiotic cells (Jégou et al., 2017). These desert regions likely resulted from selection acting against infertility phenotypes in hybrids.

When I expanded my comparison of the desert regions and regions of excess archaic ancestry with the putative sperm-related genes I focused on in the *Alouatta* hybrid system, I found evidence of putative sperm-related genes with SNPs both in areas of reduced and increased archaic ancestry in humans, suggesting autosomal loci associated with these putative

sperm-related genes might be complex in their patterns of introgression across systems. Specifically, I compared the list of 21 genes to four autosomal regions that are deserts across human populations on chromosomes 1, 3, 7, and 13, as well as the published list of genes known to be located in regions of elevated archaic ancestry (Sankararaman et al., 2014; 2016). I identified four of the putative sperm-related genes of interest (EPSTI1, GSTM1, PYGO2, and CFTR) within or near known deserts of archaic ancestry, and three genes (PLK4, SYCP3, and TSPYL1) within regions of excess archaic ancestry.

Within the deserts of archaic ancestry, these four genes are under purifying selection in howler monkeys, and GSTM1 and PYGO2 are located directly in a desert of archaic ancestry on chromosome 1 in humans. The putative homologs of these two genes in howler monkeys are associated with six SNPs located on the same genomic scaffold, with four SNPs showing patterns of excess *A. palliata* ancestry and three showing reduced introgression (because one SNP was an outlier for the alpha, but not the beta, parameter). However, the SNPs are located thousands of base pairs away from the putative genes of interest, and therefore it is unclear if these patterns of introgression actually reflect the patterns associated with these particular genes. These results are complex in that they do not show a clear pattern expected of barrier or adaptive loci, suggesting that different forms of selection act on these gene regions or on the genetic pathways in which genes in these regions are involved. It could also be that one or more of these SNPs are linked to adaptive or barrier loci, thus creating the mélange of introgression patterns. For the other two genes, EPSTI1 and CFTR, that are located near deserts of ancestry in humans, there are no available SNPs from the previous analysis on the *Alouatta* scaffolds that contain these genes, so I do not know the pattern of introgression of these genes in the howler monkey

hybrid zone. Future analysis using whole-genome resequencing to identify variants in these genes is necessary to determine their actual mode of introgression in the hybrid zone.

From my list of human sperm-related genes, three (PLK4, SYCP3, and TSPYL1) are located within known regions of excess archaic ancestry. TSPYL1 is under neutral evolution in howler monkeys and although it is relatively near two SNPs in my dataset (located ~153,000 bp and 207,000 bp away from coding sequences of one putative sperm-related gene; **Table 4-3**), it is unclear if the introgression patterns observed for these SNPs are actually associated with the introgression patterns of the genes. Regardless, these two SNPs show mixed patterns: one shows reduced introgression, and the other shows excess *A. palliata* ancestry and increased introgression, suggesting that genes in this region in the *Alouatta* hybrid zone may be affected by strong selective pressures in primates. Overall, the observed mixed patterns of introgression suggest a more complex relationship between autosomal loci located within deserts and regions of excess archaic ancestry in humans and those in the howler monkey hybrid zone compared to the patterns observed on the X chromosome (Baiz et al., 2020).

4.6 Conclusion

I created the first *de novo Alouatta pigra* reference genome assembly to compare putative sperm-related genes between *A. pigra* and *A. palliata* that are known to be vital for proper sperm development, function, and morphology in humans. I also leveraged existing genome-wide SNP data from the *A. pigra x A. palliata* hybrid zone to infer patterns of introgression of these genes, and human genomic maps of depleted ("deserts") and elevated archaic hominin ancestry to explore potentially common genes associated with reproductive isolation and adaptation in primates. Overall, I focused on 21 putative sperm-related genes, of which 17 show evidence of purifying selection and four are under neutral evolution. These 21 genes span 19 scaffolds in the

A. palliata genome assembly and contain 52 SNPs. The patterns of introgression of the SNPs varies, with most showing excess *A. palliata* ancestry (31/52), and split patterns of increased and reduced introgression (23/52 and 20/52, respectively). From my howler monkey dataset, I found six SNPs associated with genes located in deserts of archaic ancestry on human chromosome 1, with most showing *A. palliata* ancestry and reduced introgression. I also found two SNPs associated with genes in regions of elevated archaic ancestry in humans; however, these SNPs show contrasting patterns of increased and decreased introgression, and only one is an alpha outlier, showing excess *A. palliata* ancestry. However, with my data it is not possible to determine if the patterns of introgression of these SNPs are related to those of the actual genes. Overall, my results reveal a complex tapestry of putative sperm-related genes, their patterns of introgression in the howler monkey hybrid zone, and their candidacy as barrier or adaptive loci. A more comprehensive examination of additional autosomal sperm-related genes, as well as whole genome resequencing of multiple parental and admixed individuals with varying hybrid indexes, should provide more clarity on the role of sperm-related genes in reproductive isolation and adaptation in the howler monkey hybrid zone.

Table 4-1: Bioinformatics pipeline for building the de novo *Alouatta pigra* genome assembly.

Step	Purpose	Program	Reference	Code
1	Quality control of raw reads	fastQC v0.11.5	a	fastqc 520F_R1.fastq.gz 520F_R2.fastq.gz -o fastqc_data -t 32
2	Trim low-quality reads	Trimmomatic v0.36	b	TrimmomaticPE -phred33 520F_R1.fastq.gz 520F_R2.fastq.gz trimmed_520F_fwd_paired.fq trimmed_520F_fwd_unpaired.fq trimmed_520F_rev_paired.fq trimmed_520F_rev_unpaired.fq ILLUMINACLIP:TruSeq3-PE.fa:2:30:10 LEADING:3 TRAILING:3 SLIDINGWINDOW:5:20 MINLEN:50
3	Quality control of trimmed reads	fastQC v0.11.5	a	fastqc trimmed_520F_fwd_paired.fq trimmed_520F_rev_paired.fq -o fastqc_data -t 32
4	Short-read *de novo* genome assembly	SPAdes v3.15.1	c	spades.py -1 trimmed_520F_fwd_paired.fq -2 trimmed_520F_rev_paired.fq -o spades_output -t 32 -m 700
5	Quality assessment of genome assembly	QUAST v5.2	d	quast -o spades_output/quast_output_scaffolds spades_output/scaffolds.fasta
6	Depth of coverage determination	BBMap v0.11.9	e	bbmap.sh in1=trimmed_520F_fwd_paired.fq in2=trimmed_520F_rev_paired.fq ref=scaffolds.fasta covstats=constats.txt covhist=covhist.txt basecov=basecov.txt bincov=bincov.txt t=32

520F_R1 and 520F_R2 correspond to the names of my files containing the forward and reverse reads, respectively. fwd = forward, rev = reverse.

References for Table 4-1:

a. Andrews S. (2010). FastQC: a quality control tool for high throughput sequence data. Available online at https://www.bioinformatics.babraham.ac.uk/projects/fastqc/
b. Bolger AM, Lohse M, Usadel B. (2014). Trimmomatic: a flexible trimmer for Illumina sequence data. *Bioinformatics.* 30(15): 2114-2120. https://doi.org/10.1093/bioinformatics/btu170
c. Bankeich A, et al. (2012). SPAdes: a new genome assembly algorithm and its applications to single-cell sequencing. *J. Comput. Biol.* 19(5): 455-477. https://doi.org/10.1089%2Fcmb.2012.0021
d. Gurevich A, Saveliev V, Vyahhi N, Tesler G. (2013). QUAST: quality assessment tool for genome assemblies. *Bioinformatics.* 29(8): 1072-1075. https://doi.org/10.1093/bioinformatics/btt086

e. Bushnell, B. (2014). BBMap: a fast, accurate, splice-aware aligner. *Lawrence Berkeley National Laboratory*. LBNL Report #: LBNL-7065E. https://escholarship.org/uc/item/1h3515gn

Table 4-2: Reference genome assembly statistics comparison between *Alouatta pigra* and *A. palliata*.

	Alouatta pigra	***Alouatta palliata***
Sequencing platform	Illumina NovaSeq 6000 (150bp PE)	Illumina Hi-Seq 2500 (250bp PE)
Assembler	SPAdes	DISCOVAR
Total length (bp)	2,461,750,957	3,033,617,595
GC content (%)	40.71	40.8
Number of scaffolds	3,650,967	1,136,565
Scaffold N50	26,980	72,427
Number of contigs	139,436	1,152,695
Coverage (depth)	29.087X	43.1X

bp = base pairs, PE = paired-end.

Table 4-3: Twenty-one genes found in both *Alouatta* reference genome assemblies and analyzed for selection and SNP introgression.

The location of SNPs is relative to the *A. palliata* scaffold in which it is located. Direction (*α)* and amount (*β*) of introgression are displayed as (*α/β*). Positive alpha indicates excess *A. pigra* ancestry; negative alpha indicates excess *A. palliata* ancestry; positive beta indicates reduced introgression; negative beta indicates increased introgression; zero (0) indicates that the SNP was not an outlier for one of the parameters. Patterns of introgression of SNPs used in this study were determined using *bgc* (Gompert & Buerkle, 2012) and are relative to neutral expectations as described in detail in Baiz et al. (2019).

Human gene name abbreviation	**Human chromosomal location**	**MEGA11 *p*-value**	**Corresponding selection**	**Number of SNPs on scaffold**	**Direction (*α*) and amount (*β*) of introgression per SNP**
AHR	7p21.1	1	Purifying	2	+/- -/+
AK7	14q32.2	1	Purifying	4	+/0 -/+ -/0 +/0
ARMC2	6q21	1	Purifying	2	+/- -/0
AURKC	19q13.43	0.70	Neutral	3	-/+ -/+ -/+
BCL2	18q21.33	1	Purifying	2	+/+ -/-
CATSPER3	5q31.1	1	Purifying	3	+/+ -/+ +/+
CFAP44	3q13.2	1	Purifying	1	-/-
DMRT1	9p24.3	1	Purifying	1	-/-
DNAH11	7p15.3	0.34	Neutral	2	-/+ -/+
DNAI1	9p13.3	1	Purifying	4	-/+ -/- -/+ -/+
DNMT1, ETV5	19p13.2, 3q27.2	1	Purifying	4	-/- +/- -/- 0/-
EPHX2	8p21.2-p21.1	1	Purifying	4	0/+ 0/- 0/+ +/-
GNAO1	16q13	1	Purifying	6	-/+ -/+ -/0 +/0 +/0 -/-
GSTM1	1p13.3	1	Purifying	4	-/- -/0

					+/+ +/+
PON1, PON2	7q21.3	1	Purifying	4	+/- +/0 -/- -/-
PYGO2	1q21.3	1	Purifying	2	-/- -/+
STX2	12q24.33	0.59	Neutral	1	+/-
TEX11	Xq13.1	1	Purifying	1	-/+
TSPYL1	6q22.1	0.35	Neutral	2	0/- -/+

Chapter 5 : Conclusions

In this dissertation, I evaluated how hybridization affects sperm morphology and putative sperm-related gene introgression in a howler monkey hybrid system. While comparative sperm morphology has been a topic of interest for generations of scientists, the question of how hybridization affects sperm morphology in natural hybrid zones, where mechanisms of speciation can be observed, is rarely addressed. Importantly, my dissertation helps to fill this gap: it is the first to compare sperm morphology from multiple individuals of wild primates and the second to assess sperm morphology in a natural mammalian hybrid zone (Albrechtová et al., 2014). Additionally, for my dissertation I build the first *de novo* genome assembly for *A. pigra*, broadening primate representation in genome assemblies and of critically endangered mammals. Overall, this work provides a unique perspective into primate evolution and a genome assembly that can be used by other researchers to understand the evolution of primates.

In my first data chapter (chapter 2), I compared sperm morphology between *A. palliata* and *A. pigra*. I expected to observe sperm traits in *A. palliata* that would support the hypothesis that there is more intense sperm competition in this species. My results were consistent with this hypothesis. I found longer sperm head and midpiece lengths in *A. palliata* compared to *A. pigra* individuals. In other taxa, long sperm heads and midpieces have been shown to correlated with faster swimming speed compared to their shorter counterparts. Unfortunately, I do not have enough information to measure sperm motility in these primates yet; however, with new microscopy tools and methods that I recently started implementing, future work will include motility and sperm count parameters. Combining morphological data with sperm motility and

count data will provide a unique window into whether or not the long sperm heads and midpieces in *A. palliata* might correspond to faster sperm compared to those in *A. pigra*.

In my second data chapter (chapter 3), I analyzed sperm morphology in hybrids to determine how hybridization affects sperm in individuals with admixed genomes. I found that admixed hybrids with a mostly *A. palliata* genomic background (i.e., highly backcrossed into *A. palliata*) have sperm that are morphologically affected by hybridization. Specifically, I found that sperm head length in *A. palliata*-like hybrids resembles those observed in *A. palliata*; however, *A. palliata*-like sperm midpiece lengths are shorter and resemble those observed in *A. pigra*. Whether or not the shorter sperm midpieces reduces sperm motility in these hybrids remains unknown and is an attractive future path of exploration. In this chapter, I also compared proportions of morphologically abnormal sperm and testes between *A. palliata*, *A. pigra*, *A. palliata*-like hybrids, and *A. pigra*-like hybrids. Again, I found evidence that *A. palliata*-like hybrids are negatively affected by hybridization. In this case, *A. palliata*-like hybrids have more sperm and testicular morphological abnormalities compared to the other three groups. This suggests that introgression from *A. pigra* results in negative epistatic interactions between genes responsible for sperm and testicle morphology. It is likely that these hybrids also suffer from reduced reproductive fitness, as malformed testes likely do not produce adequate numbers of sperm, and morphologically abnormal sperm may have more difficulties surviving the female reproductive tract and finding and fertilizing an egg. To test this, it would be necessary to obtain sperm count and motility data taken from multiple collections over a period of time in both hybrids and unadmixed individuals. Information regarding copulatory behavior including mate choice and paternity analyses of any offspring produced from those females will also help to clarify this question.

In my final chapter (chapter 4), I explored putative sperm-related genes by leveraging human gene sequences and reduced representation genome-wide SNP data previously generated for this hybrid system. Specifically, I identified putative sperm-related genes between *A. palliata* and *A. pigra* and explored divergence between them. I analyzed signatures of selection on these putative sperm-related genes in howler monkeys, with the expectation that they would be under purifying selection given their importance in human sperm function. I then leveraged reduced-representation genomic SNP data previously generated for this hybrid zone to determine the patterns of introgression of genes located near SNP outliers, i.e., those that show significant directional introgression from one species to the other, and either reduced or increased introgression relative to null expectations. Only 21 of the 126 genes I focused on contained SNPs nearby, yet even then only a few were located within 9000 bp from the putative coding region of the gene itself. Regardless, the pattern of introgression of many of these SNPs showed excess *A. palliata* ancestry, and mixed results for increased and reduced introgression. Additionally, I implemented the methods used in Baiz et al. (2020) to start exploring whether or not these genes are located in human genomic regions of increased or reduced archaic ancestry where increased or reduced introgression of SNPs in the hybrid zone may suggest shared candidate adaptive or barrier loci, respectively. Interestingly, four of the genes I examined are located in and near regions of depleted Neanderthal and Denisovan ancestry in humans. The SNPs near these putative sperm-related genes show excess *A. palliata* ancestry, and several show reduced introgression, suggesting they may contribute to reproductive isolation. As such, these genes may be important in reproductive isolation across primates; however, additional testing across multiple primate hybrid systems is necessary to fully understand this pattern. I also examined three genes located in regions of elevated archaic ancestry, or excess Neanderthal and Denisovan

ancestry, in humans. The SNPs located on one of these genes show excess *A. palliata* ancestry; however, the SNPs differ in terms of increased versus reduced introgression, showing that some of these genes might be under different selective pressures in the hybrid zone. Additionally, many of the SNPs are physically far away from the genes in question, suggesting they may not share the same patterns of introgression as the SNP. Future genomic comparisons of these genes and patterns of introgression in other primate hybrid zones, such as the baboon and marmoset hybrid zones, will shed light into the importance of these "deserts" and regions of elevated archaic ancestry in driving speciation during the evolution of primates. In particular, there is genome wide variant data available for at least one baboon hybrid zone (Chiou et al., 2021) which can be used to determine parallels in genomic features of reproductive isolation and adaptation between humans, this howler monkey hybrid zone, and hybridizing Catarrhines.

In summary, I found sperm morphological differences between *Alouatta palliata* and *A. pigra* consistent with their different mating systems. I found that *A. palliata*-like hybrids are more negatively affected by hybridization because they have more sperm and testicular abnormalities than the other genetic categories (i.e., *A. palliata*, *A. pigra*, and *A. pigra*-like hybrids). Lastly, I explored some putative sperm-related genes in these two howler monkey species, including leveraging SNP data to determine the potential patterns of introgression of these genes in the hybrid zone, and I explored these sperm-related genes in the genomic architecture of human regions of elevated and reduced archaic ancestry.

Bibliography

Abbott RJ. (2017). Plant speciation across environmental gradients and the occurrence and nature of hybrid zones. *J. Syst. Evol.* 55(4): 238-258. https://doi.org/10.1111/jse.12267

Ackermann RR, et al. (2019). Hybridization in human evolution: insights from other organisms. *Evol. Anthropol.* 28(4): 189-209. https://doi.org/10.1002/evan.21787

Agostini I, Holzmann I, Di Bitetti MS. (2008). Infant hybrids in a newly formed mixed-species group of howler monkeys (*Alouatta guariba clamitans* and *Alouatta caraya*) in northeastern Argentina. *Primates.* 49(4): 304-307. https://doi.org/10.1007/s10329-008-0106-1

Albrecht T, Opletalova K, Reif J, Janousek V, Pialek L, Cramer ERA, et al. (2019). Sperm divergence in a passerine contact zone: Indication of reinforcement at the gametic level. *Evolution.* 73(2): 202-213. https://doi.org/10.1111/evo.13677

Albrechtová J, Albrecht T, Baird SJ, Macholan M, Rudolfsen G, Munclinger P, et al. (2012). Sperm-related phenotypes implicated in both maintenance and breakdown of a natural species barrier in the house mouse. *Proc. Biol. Sci.* 279(1748): 4803-4810. https://doi.org/10.1098/rspb.2012.1802

Albrechtová J, Albrecht T, Dureje L, Pallazola VA, Piálek J. (2014). Sperm morphology in two house mouse subspecies: do wild-derived strains and wild mice tell the same story? *PLoS One.* 10(3): e0120484. https://doi.org/10.1371/journal.pone.0120484

Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ. (1990). Basic local alignment search tool. *J. Mol. Biol.* 215: 403-410. https://doi.org/10.1016/s0022-2836(05)80360-2

Ålund M, Immler S, Rice AM, Qvarnstrom A. (2013). Low fertility of wild hybrid male flycatchers despite recent divergence. *Biol. Lett.* 9(3): 20130169. https://doi.org/10.1098/rsbl.2013.0169

Amaral A, Lourenço B, Marques M, Ramalho-Santos J. (2013). Mitochondria functionality and sperm quality. *Reproduction.* 146, 163-174. https://doi.org/10.1530/REP-13-0178

Anderson MJ, Dixson AF. (2002). Motility and the midpiece in primates. *Nature.* 416: 496. https://doi.org/10.1038/416496a

Anderson MJ, Nyholt J, Dixson AF. (2005). Sperm competition and the evolution of sperm midpiece volume in mammals. *J. Zool.* 267(2), 135-142. https://doi.org/10.1017/S0952836905007284

Anderson MJ, Dixson AS, Dixson AF. (2006). Mammalian sperm and oviducts are sexually selected: evidence for co-evolution. *J. Zool.* 270: 682-686. https://doi.org/10.1111/j.1469-7998.2006.00173.x

Andrews S. (2010). FastQC: A quality control tool for high throughput sequence data. Available online at http://www.bioinformatics.babraham.ac.uk/projects/fastqc

Baiz MD, Cortés-Ortiz L. (2015). Intragroup genetic relatedness in two howler monkey species (*Alouatta pigra* and *A. palliata*): implications for understanding social systems and dispersal. *Am. J. Primatol.* 77(12), 1333-1345. https://doi.org/10.1002%2Fajp.22487

Baiz MD, Tucker PK, Cortés-Ortiz L. (2019). Multiple forms of selection shape reproductive isolation in a primate hybrid zone. *Mol. Ecol.* 28(5): 1056-1069. https://doi.org/10.1111/mec.14966

Baiz MD, Tucker PK, Mueller JL, Cortés-Ortiz L. (2020). X-linked signature of reproductive isolation in humans is mirrored in a howler monkey hybrid zone. *J. Hered.* 111(5): 419-428. https://doi.org/10.1093/jhered/esaa021

Bankevich A, Nurk S, Sntipov D, Gurevich AA, et al. (2012). SPAdes: a new genome assembly algorithm and its applications to single-cell sequencing. *J. Comput. Biol.* 19(5): 455-477. https://doi.org/10.1089%2Fcmb.2012.0021

Barton NH, Hewitt GM. (1985). Analysis of hybrid zones. *Annu. Rev. Ecol. Syst.* 16: 113-148. https://doi.org/10.1146/annurev.es.16.110185.000553

Bates D, Mächler M, Bolker BM, Walker SC. (2015). Fitting linear mixed-effects models using lme4. *J. Stat. Softw.* 67(1), 1-48. https://doi.org/10.18637/jss.v067.i01

Bennison C, Hemmings N, Brookes L, Slate J, Birkhead T. (2016). Sperm morphology, adenosine triphosphate (ATP) concentration and swimming velocity: unexpected relationships in a passerine bird. *Proc. Royal Soc. B.* 283(1837), 20161558. https://doi.org/10.1098/rspb.2016.1558

Bicca-Marques JC, Prates HM, Aguiar FRC, Jones CB. (2008). Survey of *Alouatta caraya*, the black-and-gold howler monkey, and *Alouatta guariba clamitans*, the brown howler monkey,

in a contact zone, State of Rio Grande do Sul, Brazil: evidence for hybridization. *Primates.* 49: 246-252. https://doi.org/10.1007/s10329-008-0091-4

Bímová BV, Macholán M, Dureje L, Bímová KB, Martincová I, Piálek J. (2020). Sperm quality, aggressiveness and generation turnover facilitate unidirectional Y chromosome introgression across the European house mouse hybrid zone. *Heredity.* 125. 200-211. https://doi.org/10.1038/s41437-020-0330-z

Birkhead TR, Pizzari T. (2002). Postcopulatory sexual selection. *Nat. Rev. Genet.* 3(4): 262-273. https://doi.org/10.1038/nrg774

Bolger AM, Lohse M, Usadel B. (2014). Trimmomatic: a flexible trimmer for Illumina sequence data. *Bioinformatics*. 30(15): 2114-2120. https://doi.org/10.1093/bioinformatics/btu170

Bolin I. (1981). Male parental behavior in black howler monkeys (*Alouatta palliata pigra*) in Belize and Guatemala. *Primates.* 22(3), 349-360. https://doi.org/10.1007/BF02381575

Buerkle CA. (2005). Maximum-likelihood estimation of a hybrid index based on molecular markers. *Mol. Ecol. Notes.* 5(3): 684-687. https://doi.org/10.1111/j.1471-8286.2005.01011.x

Bushnell B. (2014). BBMap: a fast, accurate, splice-aware aligner. *Lawrence Berkeley National Laboratory*. LBNL Report #: LBNL-7065E. Retrieved from https://escholarship.org/uc/item/1h3515gn

Campbell P, Nachman MW. (2014). X-Y interactions underlie sperm head abnormality in hybrid male house mice. *Genetics.* 196(4): 1231-1240. https://doi.org/10.1534/genetics.114.161703

Carvalho FM, Arakaki PR, Nichi M, Muniz JAPC, Duarte JMB, Valle RR. (2014). Evaluation of sperm quality in successive regular collections from captive black-and-gold howler monkeys (*Alouatta caraya*). *Anim. Reprod.* 11(1), 11-18. https://doi.org/10.1007/s10764-018-0056-4

Castillo A, Marzoni M, Pirone A, Romboli I. (2012). Histological observations in testes of hybrids of *Gallus gallus* x *Phasianuns colchicus. Avian Biol. Res.* 5(1): 21-30. https://doi.org/10.3184/175815512X13267362122460

Chandley AC, Jones RC, Dott HM, Allen WR, Short RV. (1974). Meiosis in interspecific equine hybrids. I. The male mule (*Equus asinus x E. caballus*) and hinny (*E. caballus x E. asinus*). *Cytogenet. Cell Genet.* 13(4): 330-341. https://doi.org/10.1159/000130284

Charpentier MJE, Fontaine MC, Cherel E, Renoult JP, Jenkins T, Benoit L, Barthès N, Alberts SC, Tung J. (2012). Genetic structure in a dynamic baboon hybrid zone corroborates

behavioural observations in a hybrid population. *Mol. Ecol.* 21(3): 715-731. https://doi.org/10.1111/j.1365-294x.2011.05302.x

Chen PS, Stumm-Zollinger E, Aigaki T, Balmer J, Bienz M, Böhlen P. (1988). A male accessory gland peptide that regulates reproductive behavior of female D. melanogaster. *Cell.* 54: 291-298. https://doi.org/10.1016/0092-8674(88)90192-4

Chiou KL, Bergey CM, Burrell AS, Disotell TR, Rogers J, Jolly CJ, Phillips-Conroy JE. (2021). Genome-wide ancestry and introgression in a Zambian baboon hybrid zone. *Mol. Ecol.* 30(8): 1907-1920. https://doi.org/10.1111/mec.15858

Civetta A, Ranz JM. (2019). Genetic factors influencing sperm competition. *Front. Genet.* 10: 820. https://doi.org/10.3389/fgene.2019.00820

Clark ME, O'Hara FP, Chawla A, Werren JH. (2010). Behavioral and spermatogenic hybrid male breakdown in *Nasonia*. *Heredity.* 104: 289-301. https://doi.org/10.1038/hdy.2009.152

Comizzoli P, Holt WV. (2022). Recent progress in spermatology contributing to the knowledge and conservation of rare and endangered species. *Annu. Rev. Anim. Biosci.* 10, 469-490. https://doi.org/10.1146/annurev-animal-020420-040600

Corewyn LC. (2015). Dominance, access to females, and mating success among Coresident male mantled howlers (*Alouatta palliata*) at La Pacifica, Costa Rica. *Am. J. Primatol.* 77, 388-400. https://doi.org/10.1002/ajp.22355

Cortés-Ortiz L, Baiz MD, Hermida-Lagunes J, García-Orduña F, et al. (2019). Reduced introgression of sex chromosome markers in the Mexican howler monkey (*Alouatta palliata* x *A. pigra*) hybrid zone. *Int. J. Primatol.* 40, 114-131. https://doi.org/10.1007/s10764-018-0056-4

Cortés-Ortiz L, Bermingham E, Rico C, Rodríguez-Luna E, Sampaio I, Ruiz-García M. (2003). Molecular systematics and biogeography of the Neotropical monkey genus, *Alouatta*. *Mol. Phylogenet. Evol.* 26(1), 64-81. https://doi.org/10.1016/s1055-7903(02)00308-1

Cortés-Ortiz L, Duda Jr. TF, Canales-Espinosa D, García-Orduña F, Rodríguez-Luna E, Bermingham E. (2007). Hybridization in large-bodied New World primates. *Genetics.* 176(4), 2421-2425. https://doi.org/10.1534/genetics.107.074278

Cowell F. (2023). 100 years of Haldane's rule. *J. Evol. Biol.* 36(2): 337-346. https://doi.org/10.1111%2Fjeb.14112

Coyne JA, Orr HA. (2004). *Speciation.* Sunderland, Mass: Sinauer Associates.

Cramer ERA, Ålund M, McFarlane SE, Johnsen A, Qvarnström A. (2016). Females discriminate against heterospecific sperm in a natural hybrid zone. *Evolution*. 70(8): 1844-1855. https://doi.org/10.1111/evo.12986

Cramer ERA, Gronstol G, Maxwell L, Kovach AI, Lifjeld JT. (2021). Sperm length divergence as a potential prezygotic barrier in a passerine hybrid zone. *Ecol. Evol*. 11(14): 9489-9497. https://doi.org/10.1002/ece3.7768

Cunningham F, Allen JE, Allen J, et al. (2022). Ensembl 2022. *Nucleic Acids Res.* 50(1): D988-D995. https://doi.org/10.1093/nar/gkab1049

Cutter AD. (2023). Speciation and development. *Evol. Dev.* 25(4-5): 289-327. https://doi.org/10.1111/ede.12454

Dapper AL, Wade MJ. (2016). The evolution of sperm competition genes: the effect of mating system on levels of genetic variation within and between species. *Evolution.* 70(2): 502-511. https://doi.org/10.1111%2Fevo.12848

Dapper AL, Wade MJ. (2020). Relaxed selection and the rapid evolution of reproductive genes. *Trends Genet.* 36(9): 640-649. https://doi.org/10.1016/j.tig.2020.06.014

Davis H, Sosulski N, Civetta A. (2020). Reproductive isolation caused by azoospermia in sterile male hybrids of Drosophila. *Ecol. Evol.* 10(12): 5922-5931. https://doi.org/10.1002/ece3.6329

Dias PAD, Alvarado-Serrano D, Rangel-Negrín A, Canales-Espinosa D, Cortés-Ortiz L. (2013). Landscape attributes affecting the natural hybridization of Mexican howler monkeys. In: Marsh L, Chapman C. (Eds). Primates in Fragments: developments in primatology: progress and prospects. Springer, New York, NY. pp. 423-435.

Dixson AF. (2018). Copulatory and postcopulatory sexual selection in primates. *Folia Primatol.* 89(3-4): 258-286. https://doi.org/10.1159/000488105

Dunn JC, Halenar LB, Davies TG, Cristobal-Azkarate J, et al. (2015). Evolutionary trade-off between vocal tract and testes dimensions in howler monkeys. *Curr. Biol.* 25(21), 2839-2844. https://doi.org/10.1016%2Fj.cub.2015.09.029

Durvasula A, Sankararaman S. (2020). Recovering signals of ghost archaic introgression in African populations. *Sci. Adv.* 6(7): eaax5097. https://doi.org/10.1126/sciadv.aax5097

Eberhard WG. (1990). Animal genitalia and female choice. *Am. Sci.* 78(2), 134-141. https://www.jstor.org/stable/29773943

Eberhard WG. (1996). Female control: sexual selection by cryptic female choice. Vol. 69. Princeton University Press. https://doi.org/10.2307/j.ctvs32rx1

Eberhard WG. (2009). Postcopulatory sexual selection: Darwin's omission and its consequences. *PNAS.* 106(1), 10025-10032. https://doi.org/10.1073/pnas.0901217106

Edme A, Zobac P, Korsten P, Albrecht T, Schmoll T, Krist M. (2019). Moderate heritability and low evolvability of sperm morphology in a species with high risk of sperm competition, the collared flycatcher *Ficedula albicollis*. *J. Evol. Biol.* 32(3): 205-216. https://doi.org/10.1111/jeb.13404

Engels WR, Preston CR. (1979). Hybrid dysgenesis in Drosophila melanogaster: the biology of female and male sterility. *Genetics.* 92(1): 161-174. https://doi.org/10.1093/genetics/92.1.161

Fawcett DW. (1970). A comparative view of sperm ultrastructure. *Biol. Reprod.* 2(2), 90-127. https://doi.org/10.1095/biolreprod2.Supplement_2.90

Firman RC, Garcia-Gonzales F, Thyer E, Wheeler S, Yamin Z, Yuan M, Simmons LW. (2015). Evolutionary change in testes tissue composition among experimental populations of house mice. *Evolution*. 69(3): 848-855. https://doi.org/10.1111/evo.12603

Firman RC, Garcia-Gonzalez F, Simmons LW, André GI. (2018). A competitive environment influences sperm production, but not testes tissue composition, in house mice. *J. Evol. Biol.* 31(11), 1647-1654. https://doi.org/10.1111/jeb.13360

Firman RC, Gasparini C, Manier MK, Pizzari T. (2017). Postmating female control: 20 years of cryptic female choice. *Trends Ecol. Evol.* 32(5), 368-382. https://doi.org/10.1016/j.tree.2017.02.010

Firman RC, Simmons LW. (2010a). Experimental evolution of sperm quality via postcopulatory sexual selection in house mice. *Evolution.* 64(5): 1245-1256. https://doi.org/10.1111/j.1558-5646.2009.00894.x

Firman RC, Simmons LW. (2010b). Sperm midpiece length predicts sperm swimming velocity in house mice. *Biol. Lett.* 6(4), 513-516. https://doi.org/10.1098%2Frsbl.2009.1027

Firman RC. (2020). Of mice and women: advances in mammalian sperm competition with a focus on the female perspective. *Phil. Trans. R. Soc. B.* 375(1813), 20200082. https://doi.org/10.1098%2Frstb.2020.0082

Fisher HS, Jacobs-Palmer E, Lassance JM, Hoekstra HE. (2016). The genetic basis and fitness consequences of sperm midpiece size in deer mice. *Nat. Commun.* 7, 13652. https://doi.org/10.1038/ncomms13652

Fitzpatrick JL, Bridge CD, Snook RR. (2020). Repeated evidence that the accelerated evolution of sperm is associated with their fertilization function. *Proc. R. Soc. B.* 287, 20201286. https://doi.org/10.1098/rspb.2020.1286

Fitzpatrick JL, Montgomerie R, Desjardins JK, Stiver KA, Kolm N, Balshine S. (2009). Female promiscuity promotes the evolution of faster sperm in cichlid fishes. *PNAS.* 106(4), 1128-1132. https://doi.org/10.1073/pnas.0809990106

Flores-Herrera H, Acuña-Hernández DG, Rivera-Rebolledo JA, et al. (2012). Effect of increasing trypsin concentrations on seminal coagulum dissolution and sperm parameters in spider monkeys (*Ateles geoffroyi*). *Theriogenology.* 78(3), 612-619. https://doi.org/10.1016/j.theriogenology.2012.03.007

Fujiwara Y, Ogonuki N, Inoue K, Ogura A, Handel MA, Noguchi J, Kunieda T. (2013). t-SNARE Syntaxin2 (STX2) is implicatedin intracellular transport of sulfoglycolipids during meiotic prophase in mouse spermatogenesis. *Biol. Reprod.* 88(6): 141, 1-9. https://doi.org/10.1095%2Fbiolreprod.112.107110

Gage MJG. (1998). Mammalian sperm morphometry. *Proc. R. Soc. B.* 265(1391), 97-103. https://doi.org/10.1098/rspb.1998.0269

García-Vázquez FA, Gadea J, Matás C, Holt WV. (2016). Importance of sperm morphology during sperm transport and fertilization in mammals. *Asian J. Androl.* 18(6), 844-850. https://doi.org/10.4103%2F1008-682X.186880

Gay L, Crochet PA, Bell DA, Lenormand T. (2008). Comparing clines on molecular and phenotypic traits in hybrid zones: a window on tension zone models. *Evolution*. 62(11): 2789-2806. https://doi.org/10.1111/j.1558-5646.2008.00491.x

Geyer LB, Palumbi SR. (2007). Conspecific sperm precedence in two species of tropical sea urchins. *Evolution.* 59(1): 97-105. https://doi.org/10.1111/j.0014-3820.2005.tb00897.x

Godwin JL, Vasudeva R, Michalczyk L, Martin OY, Lumley AJ, Chapman T, Gage MJG (2017). Experimental evolution reveals that sperm competition intensity selects for longer, more costly sperm. *Evol. Lett.* 1(2), 102-113. https://doi.org/10.1002%2Fevl3.13

Gomendio M, Roldan ER. (2008). Implications of diversity in sperm size and function for sperm competition and fertility. *Int. J. Dev. Biol.* 52(5-6): 439-447. https://doi.org/10.1387/ijdb.082595mg

Gompert Z, Buerkle CA. (2009). A powerful regression-based method for admixture mapping of isolation across the genome of hybrids. *Mol. Ecol.* 18(6): 1207-1224. https://doi.org/10.1111/j.1365-294x.2009.04098.x

Gompert Z, Buerkle CA. (2010). introgress: a software package for mapping components of isolation in hybrids. *Mol. Ecol. Resour.* 10(2): 378-384. https://doi.org/10.1111/j.1755-0998.2009.02733.x

Gompert Z, Buerkle CA. (2012). bgc: Software for Bayesian estimation of genomic clines. *Mol. Ecol. Resour*. 12(6): 1168-1176. https://doi.org/10.1111/1755-0998.12009.x

Gould KG, Martin DE. (1978). Comparative morphology of primate spermatozoa using scanning electron microscopy. II. Families Cercopithecidae, Lorisidae, Lemuridae. *J. Hum. Evol.* 7(7), 637-642. https://doi.org/10.1016/S0047-2484(78)80048-7

Griswold MD. (1998). The central role of Sertoli cells in spermatogenesis. *Semin. Cell Dev. Biol.* 9(4): 411-416. https://doi.org/10.1006/scdb.1998.0203

Gu NH, Zhao WL, Wang GS, Sun F. (2019). Comparative analysis of mammalian sperm ultrastructure reveals relationships between sperm morphology, mitochondrial functions and motility. *Reprod. Biol. Endocrinol.* 17, 66. https://doi.org/10.1186/s12958-019-0510-y

Gurevich A, Saveliev V, Vyahhi N, Tesler G. (2013). QUAST: quality assessment tool for genome assemblies. *Bioinformatics.* 29(8): 1072-1075. https://doi.org/10.1093/bioinformatics/btt086

Haldane JBS. (1922). Sex ratio and unisexual sterility in hybrid animals. *J. Genet.* 12: 101-109. https://doi.org/10.1007/BF02983075

Harcourt AH, Harvey PH, Larson SG, Short RV. (1981). Testis weight, body weight and breeding system in primates. *Nature.* 293, 55-57. https://doi.org/10.1038/293055a0

Harcourt AH, Purvis A, Liles L. (1995). Sperm competition: mating system, not breeding season, affects testes size of primates. *Funct. Ecol.* 9(3), 468-476. https://doi.org/10.2307/2390011

Harris K, Nielsen R. (2016). The genetic cost of Neanderthal introgression. *Genetics*. 203(2): 881-891. https://doi.org/10.1534/genetics.116.186890

Harrison RG, Larson EL. (2014). Hybridization, introgression, and the nature of species boundaries. *J. Hered*. 105(1): 795-809. https://doi.org/10.1093/jhered/esu033

Harrison RG. (1990). Hybrid zones: windows on evolutionary process. In Futuyma, D. (Ed.), Oxford Surveys in Evolutionary Biology Vol. 7 (pp. 69-128). New York: Oxford University Press.

Harrison RG. (1993). Hybrid Zones and the Evolutionary Process. United Kingdom: Oxford University Press. pp. 1-364.

Hart MW, Guerra V. (2017). Finding genes and lineages under selection in speciation. *Mol. Ecol.* 26(14): 3587-3590. https://doi.org/10.1111/mec.14170

Hirst MA, Rodas-Martínez AZ, Milich KM, Cortés-Ortiz L. (2023). Differences in sperm morphology between *Alouatta palliata* and *A. pigra* are consistent with the intensity of sperm competition in each species. *Am. J. Primatol.* 85: e23538. https://doi.org/10.1002/ajp.23538

Ho L, Cortés-Ortiz L, Dias PA, Canales-Espinosa D, Kitchen DM, Bergman TJ. (2014). Effect of ancestry on behavioral variation in two species of howler monkeys (*Alouatta pigra* and *A. palliata*) and their hybrids. *Am. J. Primatol.* 76(9), 855-867. https://doi.org/10.1002/ajp.22273

Hopkins BR, Perry JC. (2022). The evolution of sex peptide: sexual conflict, cooperation, and coevolution. *Biol. Rev. Camb. Philos. Soc.* 97(4): 1426-1448. https://doi.org/10.1111%2Fbrv.12849

Hosken DJ, Archer CR, Mank JE. (2019). Sexual conflict. *Curr. Biol.* 29(11): R451-R455. https://doi.org/10.1016/j.cub.2019.03.052

Howard DJ, Gregory PG, Chu J, Cain ML. (1998). Conspecific sperm precedence is an effective barrier to hybridization between closely related species. *Evolution.* 52(2): 511-516. https://doi.org/10.1111/j.1558-5646.1998.tb01650.x

Howard DJ, Palumbi SR, Birge LM, Manier MK. (2009). Sperm and speciation. In: Birkhead TR, Hosken DJ, Pitnick S, editors. *Sperm Biology: an evolutionary perspective.* New York: Academic Press. 367-403.

Humphries S, Evans JP, Simmons LW. (2008). Sperm competition: linking form to function. *BMC Evol. Biol.* 8, 319. https://doi.org/10.1186/1471-2148-8-319

Ignatieva EV, Osadchuk AV, Kleshchev MA, Bogomolov AG, Osadchuk LV. (2021). A catalog of human genes associated with pathozoospermia and functional characteristics of those genes. *Front. Genet.* 12: 662770. https://doi.org/10.3389/fgene.2021.662770

Immler S, Pitnick S, Parker GA, Durrant KL, Lüpold S, Calhim S, Birkhead TR. (2011). Resolving variation in the reproductive tradeoff between sperm size and number. *PNAS.* 108(13), 5325-5330. https://doi.org/10.1073/pnas.1009059108

IUCN 2021. *The IUCN Red List of Threatened Species. Version 2021-1*. https://www.iucnredlist.org

Jégou B, Sankararaman S, Rolland AD, Reich D, Chalmel F. (2017). Meiotic genes are enriched in regions of reduced archaic ancestry. *Mol. Biol. Evol.* 34(8): 1974-1980. https://doi.org/10.1093/molbev/msx141

Jin J, Jin N, Zheng H, Ro S, Tafolla D, Sanders KM, Yan W. (2007). Catsper3 and Catsper4 are essential for sperm hyperactivated motility and male fertility in the mouse. *Biol. Reprod.* 77(1): 37-44. https://doi.org/10.1095/biolreprod.107.060186

Jin SK, Yang WX. (2017). Factors and pathways involved in capacitation: how are they regulated? *Oncotarget.* 8(2): 3600-3627. https://doi.org/10.18632%2Foncotarget.12274

Joly D, Bazin C, Zeng LW, Singh RS. (1997). Genetic basis of sperm and testis length differences and epistatic effect on hybrid inviability and sperm motility between Drosophila simulans and D. sechellia. *Heredity (Edinb).* 78(4): 354-362. https://doi.org/10.1038/hdy.1997.58

Jones CB, Cortés-Ortiz L. (1998). Facultative polyandry in the howling monkey (*Alouatta palliata*): Carpenter was correct. *Boletín Primatológico Latinoamericano.* 7(1), 1-7.

Jones CB. (1985). Reproductive patterns in mantled howler monkeys: estrus, mate choice and copulation. *Primates.* 26(2), 130-142. https://doi.org/10.1007/BF02382013

Kahrl AF, Cox RM. (2017). Consistent differences in sperm morphology and testis size between native and introduced populations of three *Anolis* lizard species. *J. Herpetol.* 51(4), 532-537. https://doi.org/10.1670/16-184

Kahrl AF, Snook RR, Fitzpatrick JL. (2021). Fertilization mode drives sperm length evolution across the animal tree of life. *Nat. Ecol. Evol.* 5(8), 1153-1164. https://doi.org/10.1038/s41559-021-01488-y

Kanippayoor RL, Alpern JHM, Moehring AJ. (2020). A common suite of cellular abnormalities and spermatogenetic errors in sterile hybrid males of *Drosophila*. *Proc. R. Soc. B.* 287: 20192291. https://doi.org/10.1098/rspb.2019.2291

Kelaita M, Cortés-Ortiz L. (2013). Morphological variation of genetically confirmed *Alouatta pigra* x *A. palliata* hybrids from a natural hybrid zone in Tabasco, Mexico. *Am. J. Primatol.* 150(2): 223-234. https://doi.org/10.1002/ajpa.22196

Kelaita M, Dias PAD, Aguilar-Cucurachi MdS, Canales-Espinosa D, Cortés-Ortiz L. (2011). Impact of intrasexual selection on sexual dimorphism and testes size in the Mexican howler monkeys *Alouatta palliata* and *A. pigra*. *Am. J. Primatol.* 146(2): 179-187. https://doi.org/10.1002/ajpa.21559

Kim SC, Rieseberg LH. (1999). Genetic architecture of species differences in annual sunflowers: implications for adaptive trait introgression. *Genetics.* 153(2): 965-977. https://doi.org/10.1093/genetics/153.2.965

Kitchen DM, Cortés-Ortiz L, Dias PAD, Canales-Espinosa D, Bergman TJ. (2018). *Alouatta pigra* males ignore *A. palliata* loud calls: A case of failed rival recognition? *Am. J. Phys. Anthropol.* 166(2), 433-441. https://doi.org/10.1002/ajpa.23443

Knief U, Forstmeier W, Pei Y, et al. (2017). A sex-chromosome inversion causes strong overdominance for sperm traits that affect siring success. *Nat. Ecol. Evol.* 1(8): 1177-1184. https://doi.org/10.1038/s41559-017-0236-1

Kopania EEK, Watson EM, Rathje CC, Skinner BM, Ellis PJI, Larson EL, Good JM. (2022). The contribution of sex chromosome conflict to disrupted spermatogenesis in hybrid house mice. *Genetics*. 222(4): iyac151. https://doi.org/10.1093/genetics/iyac151

Kustra MC, Alonzo SH. (2023). The coevolutionary dynamics of cryptic female choice. *Evol. Lett.* 7(4): 191-202. https://doi.org/10.1093/evlett/qrad025

Kustra MC, Kahrl AF, Reedy AM, Warner DA, Cox RM. (2019). Sperm morphology and count vary with fine-scale changes in local density in a wild lizard population. *Oecologia.* 191, 555-564. https://doi.org/10.1007/s00442-019-04511-z

Liang J, Hodge JM, Sharakhov IV. (2021). Asymmetric phenotypes of sterile hybrid males from reciprocal crosses between species of the *Anopheles gambiae* complex. *Front. Ecol. Evol.* 9: 660207. https://doi.org/10.3389/fevo.2021.660207

Losdat S, Helfenstein F. (2018). Relationships between sperm morphological traits and sperm swimming performance in wild Great Tits (*Parus major*). *J. Ornithol.* 159(3), 805-814. https://doi.org/10.1007/s10336-018-1539-y

Loyau A, Gomez D, Moureau B, Théry M, Hart NS, Jalme MS, Bennett ATD, Sorci G. (2007). Iridescent structurally based coloration of eyespots correlates with mating success in peacocks. *Behav. Ecol.* 18(6): 1123-1131. https://doi.org/10.1093/beheco/arm088

Lüdecke D. (2018). sjstats: statistical functions for regression models. Zenodo. https://doi.org/10.5281/zenodo.1284472

Lüpold S, Calhim S, Immler S, Birkhead TR. (2009). Sperm morphology and sperm velocity in passerine birds. *Proc. R. Soc. B.* 276(1659), 1175-1181. https://doi.org/10.1098%2Frspb.2008.1645

Lüpold S, de Boer RA, Evans JP, Tomkins JL, Fitzpatrick JL. (2020). How sperm competition shapes the evolution of testes and sperm: a meta-analysis. *Phil. Trans. R. Soc. B.* 375(1813), 20200064. https://doi.org/10.1098/rstb.2020.0064

Lüpold S, Fitzpatrick JL. (2015). Sperm number trumps sperm size in mammalian ejaculate evolution. *Proc. R. Soc. B.* 282(1819), 20152122. https://doi.org/10.1098/rspb.2015.2122

Lüpold S, Pitnick S. (2018). Sperm form and function: what do we know about the role of sexual selection? *Reprod.* 155(5), R229-R243. https://doi.org/10.1530/REP-17-0536

Lüpold S, Simmons LW, Grueter CC. (2019). Sexual ornaments but not weapons trade off against testes size in primates. *Proc. Biol. Sci.* 286(1900): 20182542. https://doi.org/10.1098%2Frspb.2018.2542

Maan ME, Seehausen O. (2011). Ecology, sexual selection, and speciation. *Ecol. Lett.* 14(6): 591-602. https://doi.org/10.1111/j.1461-0248.2011.01606.x

Magris M. (2021). Strategic adjustment of ejaculate quality in response to variation of the socio-sexual environment. *Behav. Ecol. Sociobiol.* 75: 91. https://doi.org/10.1007/s00265-021-03032-1

Malo AF, Gomendio M, Garde J, Lang-Lenton B, Soler AJ, Roldan ER. (2006). Sperm design and sperm function. *Biol. Lett.* 2(2), 246-249. https://doi.org/10.1098%2Frsbl.2006.0449

Malone JH, Chrzanowski TH, Michalak P. (2007). Sterility and gene expression in hybrid males of *Xenopus laevis* and *X. muelleri. PLoS One.* 2(8): e781. https://doi.org/10.1371/journal.pone.0000781

Mank JE. (2017). Population genetics of sexual conflict in the genomic era. *Nat. Rev. Genet.* 18: 721-730. https://doi.org/10.1038/nrg.2017.83

Martinez G, Coutton C, Loeuillet C, et al. (2022). Oligogenic heterozygous inheritance of sperm abnormalities in mouse. *Elife.* 11: e75373. https://doi.org/10.7554/elife.75373

Martinez G, Garcia C. (2020). Sexual selection and sperm diversity in primates. *Mol. Cell. Endocrinol.* 518, 110974. https://doi.org/10.1016/j.mce.2020.110974

Massart A, Lissens W, Tournaye H, Stouffs K. (2012). Genetic causes of spermatogenic failure. *Asian J. Androl.* 14(1): 40-48. https://doi.org/10.1038%2Faja.2011.67

Melo-Carrillo A, Dunn JC, Cortés-Ortiz L. (2020). Low genetic diversity and limited genetic structure across the range of the critically endangered Mexican howler monkey (*Alouatta palliata mexicana*). *Am. J. Primatol.* 82:e23160. https://doi.org/10.1002/ajp.23160

Mendelson TC, Safran RJ. (2021). Speciation by sexual selection: 20 years of progress. *Trends Ecol. Evol.* 36(12): 1153-1163. https://doi.org/10.1016/j.tree.2021.09.004

Møller AP. (1988). Ejaculate quality, testes size and sperm competition in primates. *J. Hum. Evol.* 17(5), 479-488. https://doi.org/10.1016/0047-2484(88)90037-1

Møller AP. (1989). Ejaculate quality, testes size and sperm production in mammals. *Funct. Ecol.* 3(1): 91-96. https://doi.org/10.2307/2389679 \

Møller AP, Höglund J. (1991). Patterns of fluctuating asymmetry in avian feather ornaments: implications for models of sexual selection. *Proc. R. Soc. B.* 245(1312): 1-5. https://doi.org/10.1098/rspb.1991.0080

Moreland RB, Richardson ME, Lamberski N, Long JA. (2001). Characterizing the reproductive physiology of the male southern black howler monkey, *Alouatta caraya*. *J. Androl.* 22(3), 395-403. https://doi.org/10.1002/J.1939-4640.2001.TB02195.X

Morrow E, Gage M. (2001). Artificial selection and heritability of sperm length in *Gryllus bimaculatus. Heredity.* 87: 356-362. https://doi.org/10.1046/j.1365-2540.2001.00921.x

Mortimer D. (2018). The functional anatomy of the human spermatozoon: relating ultrastructure and function. *Mol. Hum. Reprod.* 24(12), 567-592. https://doi.org/10.1093/molehr/gay040

Mossman J, Slate J, Humphries S, Birkhead T. (2009). Sperm morphology and velocity are genetically codetermined in the zebra finch. *Evolution*. 63(10): 2730-2737. https://doi.org/10.1111/j.1558-5646.2009.00753.x

Nakazato C, Yoshizawa M, Isobe K, Kusakabe KT, et al. (2015). Morphological characterization of spermatozoa of the night monkey. *J. Mamm. Ova Res.* 32(1): 37-40. https://doi.org/10.1274/jmor.32.37

Nei M, Kumar S. (2000). *Molecular Evolution and Phylogenetics.* Oxford University Press, New York. pp. 52-62.

Nikolettos N, Küpker W, Demirel C, Schöpper B, Blasig C, Sturm R, Felberbaum R, Bauer O. Diedrich K, Al-Hasani S. (1999). Fertilization potential of spermatozoa with abnormal morphology. *Hum. Reprod.* 14(1): 47-70. https://doi.org/10.1093/humrep/14.suppl_1.47

Oka A, Mita A, Takada Y, Koseki H, Shiroishi T. (2010). Reproductive isolation in hybrid mice due to spermatogenesis defects at three meiotic stages. *Genetics.* 186(1): 339-351. https://doi.org/10.1534%2Fgenetics.110.118976

Oliveira KG, Santos RR, Leão DL, Queiroz HL, Paim FP, Vianez-Júnior JL, et al. (2016). Testicular biometry and semen characteristics in captive and wild squirrel monkey species (*Saimiri* sp.). *Theriogenology.* 86(3), 879-887. https://doi.org/10.1016/j.theriogenology.2016.03.009

Olson GE, Winfrey VP. (1990). Mitochondria-Ccytoskeleton interactions in the sperm midpiece. *J. Struct. Biol.* 103(1), 13-22. https://doi.org/10.1016/1047-8477(90)90081-m

Omotoriogun TC, Laskemoen T, Rowe M, Albrecht T, Bowie RCK, Sedláček O, et al. (2016). Variation in sperm morphology among Afrotropical sunbirds. *Ibis.* 158(1), 155-166. https://doi.org/10.1111/ibi.12334

Orr HA, Turelli M. (2001). The evolution of postzygotic isolation: accumulating Dobzhansky-Müller incompatibilities. *Evolution.* 55(6): 1085-1094. https://doi.org/10.1554/0014-3820(2001)055[1085:TEOPIA]2.0.CO;2

Palumbi SR. (2009). Speciation and the evolution of gamete recognition genes: pattern and process. *Heredity*. 102: 66-76. https://doi.org/10.1038/hdy.2008.104

Panhuis TM, Butlin R, Zuk M, Tregenza T. (2001). Sexual selection and speciation. *Trends Ecol. Evol.* 16(7): 364-371. https://doi.org/10.1016/S0169-5347(01)02160-7

Papaioannou MD, Pitetti JL, Ro S, Park C, et al. (2009). Sertoli cell Dicer is essential for spermatogenesis in mice. *Dev. Biol.* 326(1): 250-259. https://doi.org/10.1016/j.ydbio.2008.11.011

Parker GA, Partridge L. (1998). Sexual conflict and speciation. *Phil. Trans. R. Soc. Lond. B.* 353(1366), 261-274. https://doi.org/10.1098%2Frstb.1998.0208

Parker GA, Pizzari T. (2010). Sperm competition and ejaculate economics. *Biol. Rev.* 85(4), 897-934. https://doi.org/10.1111/j.1469-185x.2010.00140.x

Parker GA. (1979). Sexual conflict over mating and fertilization: a review. *Philos. Trans. R. Soc. Lond. B. Biol. Sci.* 361(1466): 235-259. https://doi.org/10.1098%2Frstb.2005.1785

Parker GA. (1970). Sperm competition and its evolutionary consequences in the insects. *Biol. Rev*. 45(4), 525–567. https://doi.org/10.1111/j.1469-185X.1970.tb01176.x

Parker GA. (1982). Why are there so many tiny sperm? Sperm competition and the maintenance of two sexes. *J. Theor. Biol.* 96(2), 281-294. https://doi.org/10.1016/0022-5193(82)90225-9

Parker GA. (1993). Sperm competition games: sperm size and sperm number under adult control. *Proc. R. Soc. B.* 253(1338), 245-254. https://doi.org/10.1098/rspb.1993.0110

Piomboni P, Focarelli R, Stendardi A, Ferramosca A, Zara V. (2011). The role of mitochondria in energy production for human sperm motility. *Int. J. Androl.* 35(2), 109-124. https://doi.org/10.1111/j.1365-2605.2011.01218.x

Pitnick S, Hosken DJ, Birkhead TR. (2009). Sperm morphological diversity. In: Birkhead TR, Hosken DJ, Pitnick S, editors. *Sperm Biology: an evolutionary perspective.* New York: Academic Press. 69-149.

Poignet M, Baránková L, Reif J, Stopka P, Stopková R, Frolikova M, Cramer ERA, Johnsen A, Kverek P, Osiejuk TS, Komrskova K, Albrecht T, Reifová R. (2022). Sperm morphology and performance in relation to postmating prezygotic isolation in two recently diverged passerine species. *Sci. Rep.* 12: 22275. https://doi.org/10.1038/s41598-022-26101-5

Pound N, Gage MJG. (2004). Prudent sperm allocation in Norway rats, *Rattus norvegicus*: a mammalian model of adaptive ejaculate adjustment. *Anim. Behav.* 68(4): 819-823. https://doi.org/10.1016/j.anbehav.2004.02.004

Ratomponirina C, Andrianivo J, Rumpler Y. (1982). Spermatogenesis in several intra- and interspecific hybrids of the lemur (*Lemur*). *J. Reprod. Fert.* 66: 717-721. https://doi.org/10.1530/jrf.0.0660717

Reinhold K. (2000). Maintenance of a genetic polymorphism by fluctuating selection on sex-limited traits. *J. Evol. Biol.* 13(6): 1009-1014. https://doi.org/10.1046/j.1420-9101.2000.00229.x

Richard RD, LaChance LE, Proshold FI. (1975). An ultrastructural study of sperm in sterile hybrids from crosses of *Heliothis virescens* and *Heliothis subflexa*. *Ann. Entomol. Soc. Am.* 68(1): 35-39. https://doi.org/10.1093/aesa/68.1.35

Rieseberg LH, Whitton J, Gardner K. (1999). Hybrid zones and the genetic architecture of a barrier to gene flow between two sunflower species. *Genetics*. 152(2): 713-727. https://doi.org/10.1093/genetics/152.2.713

Ritchie MG. (2007). Sexual selection and speciation. *Annu. Rev. Ecol. Evol. Syst.* 38: 79-102. https://doi.org/10.1146/annurev.ecolsys.38.091206.095733

Roldan ERS. (2019). Sperm competition and the evolution of sperm form and function in mammals. *Reprod. Domest. Anim.* 54(4), 14-21. https://doi.org/10.1111/rda.13552

Rowe M, Albrecht T, Cramer ER, Johnsen A, Laskemoen T, Weir JT, Lifjeld JT. (2015). Postcopulatory sexual selection is associated with accelerated evolution of sperm morphology. *Evolution.* 69(4), 1044-1052. https://doi.org/10.1111/evo.12620

Rowe M, Veerus L, Trosvik P, Buckling A, Pizzari T. (2020). The reproductive microbiome: an emerging driver of sexual selection, sexual conflict, mating systems, and reproductive isolation. *Trends Ecol. Evol.* 35(3), 220-234. https://doi.org/10.1016/j.tree.2019.11.004

Rowe M, Whittington E, Borziak K, Ravinet M, Eroukhmanoff F, Saetre GP, Dorus S. (2019). Molecular diversification of the seminal fluid proteome in a recently diverged passerine species pair. *Mol. Biol. Evol.* 37(2): 488-506. https://doi.org/10.1093/molbev/msz235

RStudio Team (2020). RStudio: integrated development for R. RStudio, PBC, Boston, MA http://www.rstudio.com/

Rudolfsen G, Figenschou L, Folstad I, Kleven O. (2008). Sperm velocity influence paternity in the Atlantic cod (*Gadus morhua* L.). *Aquac. Res.* 39(2): 212-216. https://doi.org/10.1111/j.1365-2109.2007.01863.x

Ryan SF, Deines JM, Scriber JM, Pfrender ME, Jones SE, Emrich SJ, Hellmann J. (2018). Climate-mediated hybrid zone movement revealed with genomics, museum collection, and simulation modeling. *PNAS.* 115(10), E2284-E2291. https://doi.org/10.1073/pnas.1714950115

Presgraves DC. (2018). Evaluating genomic signatures of "the large X-effect" during complex speciation. *Mol. Ecol.* 27(19): 3822-3830. https://doi.org/10.1111%2Fmec.14777

Saacke RG. (2008). Sperm morphology: its relevance to compensable and uncompensable traits in semen. *Theriogenology.* 70(3): 473-478. https://doi.org/10.1016/j.theriogenology.2008.04.012

Sampaio WV, Oliveira KG, Leão DL, Caldas-Bussiere MC, Queiroz HL, et al. (2017). Morphologic analysis of sperm from two neotropical primate species: comparisons between the squirrel monkeys *Saimiri collinsi* and *Saimiri vanzolinii*. *Zygote.* 25(2), 141-148. https://doi.org/10.1017/s0967199416000411

Sankararaman S, Mallick S, Dannemann M, Prüfer K, Kelso J, Pääbo S, Patterson N, Reich D. (2014). The genomic landscape of Neanderthal ancestry in present-day humans. *Nature*. 507: 354-357. https://doi.org/10.1038/nature12961

Sankararaman S, Mallick S, Patterson N, Reich D. (2016). The combined landscape of Denisovan and Neanderthal ancestry in present-day humans. *Curr. Biol.* 26(9): 1241-1247. https://doi.org/10.1016/j.cub.2016.03.037

Schneider CA, Rasband WS, Eliceiri KW. (2012). NIH Image to ImageJ: 25 years of image analysis. *Nat. Methods.* 9(7), 671-675. https://doi.org/10.1038/nmeth.2089

Schwahn DJ, Wang RJ, White MA, Payseur BA. (2018). Genetic dissection of hybrid male sterility across stages of spermatogenesis. *Genetics.* 210(4): 1453-1465. https://doi.org/10.1534/genetics.118.301658

Servedio MR, Boughman JW. (2017). The role of sexual selection in local adaptation and speciation. *Ann. Rev. Ecol. Evol. Syst.* 48: 85-109. https://doi.org/10.1146/annurev-ecolsys-110316-022905

Sequencher® version 5.4.6 DNA sequence analysis software, Gene Codes Corporation, Ann Arbor, MI USA http://www.genecodes.com

Sharpe RM, McKinnell C, Kivlin C, Fisher JS. (2003). Proliferation and functional maturation of Sertoli cells, and their relevance to disorders of testis function in adulthood. *Reproduction*. 125(6): 769-784. https://doi.org/10.1530/rep.0.1250769

Shen X, Song S, Li C, Zhang J. (2021). Synonymous mutations in representative yeast genes are mostly strongly non-neutral. *Nature*. 606: 725-731. https://doi.org/10.1038/s41586-022-04823-w

Shimazaki M, Wittayarat M, Sambuu R, Sugita A, Kawaguchi M, Hirata M, Tanihara F, Takagi M, Taniguchi M, Otoi T, Sato Y. (2022). Disruption of cell proliferation and apoptosis

balance in the testes of crossbred cattle-yaks affects spermatogenic cell fate and sterility. *Reprod. Dom. Anim.* 57: 999-1006. https://doi.org/10.1111/rda.14166

Short RV. (1979). Sexual selection and its component parts, somatic and genital selection, as illustrated by man and the great apes. *Adv. Study Behav.* 9, 131-158. https://doi.org/10.1016/S0065-3454(08)60035-2

Simmons LW, Moore AJ. (2009). Evolutionary quantitative genetics of sperm. In: Birkhead TR, Hosken DJ, Pitnick S, editors. *Sperm Biology: an evolutionary perspective.* New York: Academic Press. 405-434.

Simpson JL, Humphries S, Evans JP, Simmons LW, Fitzpatrick JL. (2013). Relationships between sperm length and speed differ among three internally and three externally fertilizing species. *Evolution.* 68(1), 92-104. https://doi.org/10.1111/evo.12199

Smith JM. (1991). Theories of sexual selection. *Trends Ecol. Evol.* 6(5): 146-151. https://doi.org/10.1016/0169-5347(91)90055-3

Snook RR. (2005). Sperm in competition: not playing by the numbers. *Trends Ecol. Evol.* 20(1): 46-53. https://doi.org/10.1016/j.tree.2004.10.011

Stecher G, Tamura K, Kumar S. (2020). Molecular Evolutionary Genetics Analysis (MEGA) for macOS. *Mol. Biol. Evol.* 37:1237-1239.

Steinberg ER, Nieves M, Ascunce MS, Palermo AM, Mudry MD. (2009). Morphological and genetic characterization of *Saimiri boliviensis. Int. J. Primatol.* 30: 29-41. https://doi.org/10.1007/s10764-008-9328-8

Steinberg ER, Nieves M, Mudry MD. (2014). Multiple sex chromosome systems in howler monkeys (Platyrrhini, *Alouatta*). *Comp. Cytogenet.* 8(1), 43-69. https://doi.org/10.3897%2FCompCytogen.v8i1.6716

Steinberg ER, Sestelo AJ, Ceballos MB, Wagner V, Palermo AM, Mudry MD. (2019). Sperm morphology in Neotropical primates. *Animals (Basel).* 9(10), 839. https://doi.org/10.3390%2Fani9100839

Steiner CC, Ryder OA. (2013). Characterization of Prdm9 in equids and sterility in mules. *PLoS One.* 8(4): e61746. https://doi.org/10.1371/journal.pone.0061746

Stergachis AB, Haugen E, Shafer A, Fu W, Vernot B, Reynolds A, Raubitschek A, Ziegler S, LeProust EM, Akey JM, Stamatoyannopoulos JA. (2013). Exonic transcription factor binding

directs codon choice and affects protein evolution. *Science*. 342(6164): 1367-1372. https://doi.org/10.1126%2Fscience.1243490

Støstad HN, Johnsen A, Lifjeld JT, Rowe M. (2018). Sperm head morphology is associated with sperm swimming speed: A comparative study of songbirds using electron microscopy. *Evolution.* 72(9), 1918-1932. https://doi.org/10.1111/evo.13555

Stumpf RM, Martinez-Mota R, Milich KM, Righini N, Shattuck MR. (2011). Sexual conflict in primates. *Evol. Anthropol.* 20, 62–75. https://doi.org/10.1002/evan.20297

Svensson EI. (2018). Eco-evolutionary dynamics of sexual selection and sexual conflict. *Funct. Ecol.* 33(1), 60-72. https://doi.org/10.1111/1365-2435.13245

Swanson WF, Valle RR, Carvalho FM, Arakaki PR, Rodas-Martínez AZ, Muniz J, García-Herreros M. (2016). Sperm morphology assessment in captive Neotropical primates. *Reprod. Domest. Anim.* 51(4), 623-627. https://doi.org/10.1111/rda.12711

Swanson WJ, Vacquier VD. (2002). The rapid evolution of reproductive proteins. *Nat. Rev. Genet.* 3(2): 137-144. https://doi.org/10.1038/nrg733

Szymura JM, Barton NH. (1986). Genetic analysis of a hybrid zone between the fire-bellied toads, *Bombina bombina* and *B. variegata*, near Cracow in southern Poland. *Evolution*. 40(6): 1141-1159. https://doi.org/10.1111/j.1558-5646.1986.tb05740.x

Takihara H, Cosentino MJ, Sakatoku J, Cockett ATK. (1987). Significance of testicular size measurement in andrology: II. Correlation of testicular size with testicular function. *J. Urol.* 137(3): 416-419. https://doi.org/10.1016/s0022-5347(17)44053-5

Tamura K, Stecher G, Kumar S. (2021). MEGA 11: Molecular Evolutionary Genetics Analysis Version 11. *Mol. Biol. Evol.* https://doi.org/10.1093/molbev/msab120

Taylor SA, Larson EL. (2019). Insights from genomes into the evolutionary importance and prevalence of hybridization in nature. *Nat. Ecol. Evol*. 3(2): 170-177. https://doi.org/10.1038/s41559-018-0777-y

Temple-Smith PD, Ravichandran A, Horta Nunez FE. (2018). Sperm: comparative vertebrate. In: Encyclopedia of Reproduction (2nd Edition), Vol. 6. Ed: MK Skinner, pp 210-220. Academic Press: Elsevier http://dx.doi.org/10.1016/B978-0-12-809633-8.20558-X

Thonhauser KE, Thoß M, Musolf K, Klaus T, Penn DJ. (2013). Multiple paternity in wild house mice (*Mus musculus musculus*): effects on offspring genetic diversity and body mass. *Ecol. Evol.* 4(2): 200-209. https://doi.org/10.1002%2Fece3.920

Tourmente M, Gomendio M, Roldan ER. (2011). Sperm competition and the evolution of sperm design in mammals. *BMC Evol. Biol.* 11. https://doi.org/10.1186/1471-2148-11-12

Treves A. (2001). Reproductive consequences of variation in the composition of howler monkey (*Alouatta* spp.) groups. *Behav. Ecol. Sociobiol.* 50(1), 61-71. https://doi.org/10.1007/s002650100329

Tung J, Barreiro LB. (2017). The contribution of admixture to primate evolution. *Curr. Op. Genet. Dev.* 47: 61-68. https://doi.org/10.1016/j.gde.2017.08.010

Turelli M, Barton NH, Coyne JA. (2001).Theory and speciation. *Trends Ecol. Evol.* 16(7): 330-343. https://doi.org/10.1016/S0169-5347(01)02177-2

Turner LM, Harr B. (2014). Genome-wide mapping in a house mouse hybrid zone reveals hybrid sterility loci and Dobzhansky-Müller interactions. *eLife.* 3: e02504. https://doi.org/10.7554/eLife.02504

Turner LM, Hoekstra HE. (2008) Causes and consequences of the evolution of reproductive proteins. *Int. J. Dev. Biol.* 52(5-6): 769-780. https://doi.org/10.1387/ijdb.082577lt

Turner LM, Schwahn DJ, Harr B. (2012). Reduced male fertility is common but highly variable in form and severity in a natural house mouse hybrid zone. *Evolution.* 66(2): 443-458. https://doi.org/10.1111/j.1558-5646.2011.01445.x

Uhlén M, Fagerberg L, Hallström BM, Lindskog C, et al. (2015). Tissue-based map of the human proteome. *Science*. 347(6220): 1260419. https://doi.org/10.1126/science.1260419 (proteinatlas.org)

Valle RR, Arakaki PR, Carvalho FM, Muniz JA, Leal CL, García-Herreros M. (2013a). Identification of sperm head subpopulations with defined pleiomorphic characteristics in ejaculates of captive Goeldi's monkeys (*Callimico goeldii*). *Anim. Reprod. Sci.* 137(1-2), 93-102. https://doi.org/10.1016/j.anireprosci.2012.12.007

Valle RR, Carvalho FM, Muniz JA, Leal CL, García-Herreros M. (2013b). Differential distribution of sperm subpopulations and incidence of pleiomorphisms in ejaculates of captive howling monkeys (*Alouatta caraya*). *Naturwissenschaften.* 100(10), 923-933. https://doi.org/10.1007/s00114-013-1092-8

Valle RR, Guimarães MA, Muniz JA, Barnabe RC, Vale WG. (2004). Collection and evaluation of semen from captive howler monkeys (*Alouatta caraya*). *Theriogenology.* 62(1-2), 131-138. https://doi.org/10.1016/j.theriogenology.2003.08.004

Valle RR, Nayudu PL, Leal CL, García-Herreros M. (2012). Sperm head morphometry in ejaculates of adult marmosets (*Callithrix jacchus*): a model for studying sperm subpopulations and among-donor variations. *Theriogenology.* 78(5), 1152-1165. https://doi.org/10.1016/j.theriogenology.2012.05.017

Van Belle S, Bicca-Marques JC. (2015). Insights into reproductive strategies and sexual selection in howler monkeys. In: MM Kowalewski, PA Garber, L Cortés-Ortiz, B. Urbani, D. Youlatos, Eds. *Howler monkeys, developments in primatology: Progress and prospects* (pp. 57-84). Springer Science + Business Media. https://doi.org/10.1007/978-1-4939-1960-4_3

Van Belle S, Estrada A, Strier KB, Di Fiore A. (2012). Genetic structure and kinship patterns in a population of black howler monkeys, *Alouatta pigra*, at Palenque National Park, Mexico. *Am. J. Primatol.* 74(10), 948-957. https://doi.org/10.1002/ajp.22047

Van Belle S, Estrada A, Ziegler TE, Strier KB. (2009a). Sexual behavior across ovarian cycles in wild black howler monkeys (*Alouatta pigra*): male mate guarding and female mate choice. *Am. J. Primatol.* 71(2), 153-164. https://doi.org/10.1002/ajp.20635

Van Belle S, Estrada A, Ziegler TE, Strier KB. (2009b). Social and hormonal mechanisms underlying male reproductive strategies in black howler monkeys (*Alouatta pigra*). *Horm. Behav.* 56(4), 355-363. https://doi.org/10.1016/j.yhbeh.2009.08.006

Van Belle S, Estrada A. (2008). Group size and composition influence male and female reproductive success in black howler monkeys (*Alouatta pigra*). *Am. J. Primatol.* 70(6), 613-619. https://doi.org/10.1002/ajp.20534

Van Belle S, Estrada A, Strier KA. (2008). Social relationships among male *Alouatta pigra. Int. J. Primatol.* 29: 1481-1498. https://doi.org/10.1007/s10764-008-9309-y

Van Belle S, Garber PA, Estrada A, Di Fiore A. (2014). Social and genetic factors mediating male participation in collective group defence in black howler monkeys. *Anim. Behav.* 98, 7-17. https://doi.org/10.1016/j.anbehav.2014.09.023

van der Horst G, Maree L. (2013). Sperm form and function in the absence of sperm competition. *Mol. Reprod. Dev.* 81(3): 204-216. https://doi.org/10.1002/mrd.22277

Varea-Sánchez M, Bastir M, Roldan ER. (2013). Geometric morphometrics of rodent sperm head shape. *PLoS One.* 8(11), e80607. https://doi.org/10.1371/journal.pone.0080607

Varea-Sánchez M, Tourmente M, Bastir M, Roldan ER. (2016). Unraveling the sperm bauplan: relationships between sperm head morphology and sperm function in rodents. *Biol. Reprod.* 95(1), 25, 1-9. https://doi.org/10.1095/biolreprod.115.138008

Vawter L, Brown WM. (1986). Nuclear and mitochondrial DNA comparisons reveal extreme rate variation in the molecular clock. *Science*. 234(4773): 194-196. https://doi.org/10.1126/science.3018931

Wang E, Milton K. (2003). Intragroup social relationships of male *Alouatta palliata* on Barro Colorado Island, Republic of Panama. *Int. J. Primatol.* 24, 1227-1243. https://doi.org/10.1023/B:IJOP.0000005989.29238.ce

White MA, Stubbings M, Dumont BL, Payseur BA. (2012). Genetics and evolution of hybrid male sterility in house mice. *Genetics.* 191(3): 917-934. https://doi.org/10.1534/genetics.112.140251

Wilburn DB, Swanson WJ. (2016). From molecules to mating: rapid evolution and biochemical studies of reproductive proteins. *J. Proteome.* 135: 12-25. https://doi.org/10.1016/j.jprot.2015.06.007

Wildmayer SJ, Handel MA, Aylor DL. (2020). Age and genetic background modify hybrid male sterility in house mice. *Genetics.* 216(2): 585-597. https://doi.org/10.1534/genetics.120.303474

Wooley DM, Beatty RA. (1967). Inheritance of midpiece length in mouse spermatozoa. *Nature.* 215: 94-95. https://doi.org/10.1038/215094a0

World Health Organization. (2021). WHO Laboratory manual for the examination and processing of human semen. 6th Ed. WHO Press. Geneva, Switzerland. Available online: https://www.who.int/publications/i/item/9789240030787

Wünsch LK, Pfennig KS. (2013). Failed sperm development as a reproductive isolating barrier between species. *Evol. Dev.* 15(6): 458-465. https://doi.org/10.1111/ede.12054

Yang Y, Li X, Ye S, Chen X, Wang L, Qian Y, Xin Q, Li L, Gong P. (2022). Identification of genes related to sexual differentiation and sterility in embryonic gonads of Mule ducks by transcriptome analysis. *Front. Genet.* 13:1037810. https://doi.org/10.3389/fgene.2022.1037810

Yoshikawa H, Xu D, Ino Y, Yoshino T, Hayashida T, Wang J, Yazawa R, Yoshizaki G, Takeuchi Y. Hybrid sterility in fish caused by mitotic arrest of primordial germ cells. *Genetics.* 209(2): 507-521. https://doi.org/10.1534/genetics.118.300777

Yoshiki A, Moriwaki K, Sakakura T, Kusakabe M. (1993). Histological studies on male sterility of hybrids between laboratory and wild mouse strains. *Dev. Growth Differ.* 35(3): 271-281. https://doi.org/10.1111/j.1440-169X.1993.00271.x

Zhang BL, Chen W, Wang Z, Pang W, et al. (2023). Comparative genomics reveals hybrid origin of a macaque group. *Sci. Adv.* 9(22): eadd3580. https://doi.org/10.1126/sciadv.add3580

Zhang J, Kumar S, Nei M. (1997). Small-sample tests of episodic adaptive evolution: a case study of primate lysozymes. *Mol. Biol. Evol.* 14:1335-1338. https://doi.org/10.1093/oxfordjournals.molbev.a025743

Zhang J, Rosenberg HF, Nei M (1998). Positive Darwinian selection after gene duplication in primate ribonuclease genes. *PNAS* (*USA*) 95:3708-3713. https://doi.org/10.1073/pnas.95.7.3708

Zinner D, Arnold ML, Roos C. (2017). The strange blood: natural hybridization in primates. *Evol. Anthropol.* 20(3): 96-103. https://doi.org/10.1002/evan.20301

Zoonomia Consortium. (2020). A comparative genomics multitool for scientific discovery and conservation. *Nature.* 587: 240-245. https://doi.org/10.1038/s41586-020-2876-6