GENETICS, 2025, 231(3), iyaf177
https://doi.org/10.1093/genetics/iyaf177
Advance Access Publication Date: 1 September 2025
Investigation

Low mutation rate but high male-bias in the germline of a short-lived opossum

Yadira Peña-García [1,*] Richard J. Wang,[1] Muthuswamy Raveendran [2,3] R. Alan Harris,[2,3] Paul B. Samollow,[4] Jeffrey Rogers [2,3] Matthew W. Hahn [1,5]

[1]Department of Biology, Indiana University Bloomington, Bloomington, IN 47405, United States
[2]Human Genome Sequencing Center, Baylor College of Medicine, Houston, TX 77030, United States
[3]Department of Molecular and Human Genetics, Baylor College of Medicine, Houston, TX 77030, United States
[4]Department of Veterinary Integrative Biosciences, Texas A&M University, College Station, TX 77843, United States
[5]Department of Computer Science, Indiana University Bloomington, Bloomington, IN 47405, United States

*Corresponding author: Department of Biology, Indiana University Bloomington, Bloomington, IN 47405, United States. Email: ypenagar@iu.edu

Age and sex have been found to be important determinants of the mutation rate per generation in mammals, but the mechanisms underlying these factors are still unclear. One approach to distinguishing between alternative mechanisms is to study species that reproduce at very young ages, as competing hypotheses make different predictions about patterns of mutation in these organisms. Here, we study the germline mutation rate in the gray short-tailed opossum, *Monodelphis domestica*, a laboratory model species that becomes reproductively mature at less than 6 mo of age. Whole-genome sequencing of 22 trios reveals one of the lowest mutation rates per generation found in mammals thus far (0.252×10^{-8} per base pair per generation at an average parental age of 313 d), which is expected given their early reproduction. We also examine the mutation spectrum and find fewer mutations at CpG sites in opossums than in humans, consistent with the lower CpG content in the opossum genome. We observe that two-thirds of mutations are inherited from the male parent in opossums, slightly lower than the degree of male bias observed in organisms that reproduce at much older ages. Nevertheless, the very young age at reproduction in opossums suggests that ongoing spermatogonial divisions in males after puberty are not the primary driver of the observed male mutation bias. These findings contribute to a growing body of evidence that the differences between male and female germline mutation may arise from mechanisms other than cell division postpuberty.

Keywords: reproductive maturity; evolution; germline; generation time

Introduction

Germline mutations are the primary source of genetic variation and a fundamental driver of evolution. While most de novo mutations (DNMs) are neutral, some can cause heritable diseases or contribute to adaptive traits, highlighting their critical role in both evolutionary processes and human health. The first estimates of the mutation rate were derived from the study of rare Mendelian diseases in humans (Haldane 1935) and then later through the analysis of sequence divergence between species with known divergence times (Nachman and Crowell 2000; Silva and Kondrashov 2002). Advances in sequencing technologies have facilitated the direct estimation of mutation rates using pedigrees, enabling the identification of mutations arising between parents and offspring (Yoder and Tiley 2021). As pedigree sequencing proliferates, the number of species for which the mutation rate has been estimated continues to grow (e.g. Venn et al. (2014); Thomas et al. 2018; Besenbacher et al. 2019; Koch et al. 2019; Wang et al. 2020, 2022a,b; Wu et al. 2020; Bergeron et al. 2023; Suarez-Menendez et al. 2023; Armstrong et al. 2025). These studies have shown that per-generation mutation rates can vary by a factor of 40 across vertebrates (Bergeron et al. 2023).

Variation in the per-generation mutation rate has been attributed to differences in life-history traits, especially generation time (Thomas et al. 2018; Wang et al. 2022b; Bergeron et al. 2023; Zhu et al. 2025). In mammals, generation time has a large effect because both male and female parents transmit more mutations as they age (Kong et al. 2012; Goldmann et al. 2016; Wong et al. 2016; Jonsson et al. 2017). This means that species with shorter generation times will have lower per-generation mutation rates, simply because they reproduce at younger ages. In addition, mammalian fathers consistently pass on more mutations than mothers—approximately three-fourths of all mutations (de Manuel et al. 2022)—a phenomenon known as male mutation bias (Wilson Sayres and Makova 2011). This difference between the sexes has been attributed to the higher number of cell divisions, and thus DNA replication cycles, during spermatogenesis compared to oogenesis, leading to a greater incidence of replication errors in males; it is thought that the male and female germlines undergo a similar number of cell divisions before puberty (Drost and Lee 1995; Crow 2000). However, studies in humans, mice, and cats have shown the existence of a consistently strong male bias even shortly after puberty, which does not support a replication-driven hypothesis for male bias (Jonsson et al. 2017; Lindsay et al. 2019; Wang et al. 2022b).

Received on 26 April 2025; accepted on 01 August 2025

Instead, alternative hypotheses for the origin of male bias have been proposed (Gao et al. 2019; Hahn et al. 2023), though more species must be studied in order to properly evaluate alternative models.

Here, we contribute to the understanding of mammalian germline mutation rates and male mutation bias. We used pedigree sequencing data from the gray short-tailed opossum (*Monodelphis domestica*), originally native to Brazil and surrounding countries (VandeBerg and Robinson 1997), to explore the effect of paternal and maternal age on the mutation rate. Marsupials and eutherian mammals diverged approximately 160 million years ago (Luo et al. 2011), with marsupials exhibiting many distinct developmental and gestational traits compared to eutherians. While there are multiple studies of the effects of parental age and male-biased mutation among eutherians, we present the most comprehensive account in a marsupial to date. A previous study by Bergeron et al. (2023) estimated the mutation rate in *Monodelphis domestica* based on a single trio. While valuable, single-trio estimates represent a limited snapshot, as mutation rates can vary across individuals and parental ages. By analyzing a larger dataset of trios, our study offers a more complete picture of germline mutation rates in this species. These data also provide a test of how well existing mutation models, primarily developed in humans, generalize across mammals. Our results in the opossum reveal one of the lowest mutation rates per site per generation reported to date in mammals, which is consistent with expectations given the early reproduction among individuals in our study (0.5 to 1 yr). We also observe a significant male mutation bias, even among the youngest parents. Finally, our analysis of the mutation spectrum showed a difference from other mammals, especially in mutations at CpG dinucleotides. These findings provide new insights into the cellular mechanisms governing the evolution of mutation rates and sex-specific biases in mammals, and emphasize the importance of studying diverse species to understand the broad applicability of existing models.

Materials and methods

Samples and sequencing

Liver samples were collected from a total of 34 individuals that were part of 3 extended pedigrees, comprising 22 trios, of gray short-tailed opossums (*Monodelphis domestica*) kept in captivity at Texas A&M University (College Station, TX, United States). These animals are descendants of individuals collected from 1978 to 1993 from 5 locations across Brazil and Bolivia (Xiong et al. 2022). Animal care and experimental procedures were conducted in strict accordance with policies and guidelines of Texas A&M. Genomic DNA extraction was performed from the collected samples using Gentra Puregene methods and reagents, following established protocols. The extracted DNA was sheared into fragments of approximately 200 to 600 bp. Sequencing libraries were prepared as described in Wang et al. (2022b). Briefly, PCR-free libraries were generated using KAPA Hyper PCR-free reagents. The sheared DNA molecules were then purified using AMPure XP beads. The procedure next included DNA end-repair and 3′ adenylation followed by addition of barcoded adapters, generating library inserts with an average length of 426 bp. Whole-genome sequencing of the prepared libraries was performed on Illumina Hi-Seq X instruments, following manufacturer's recommendation with minor modifications. We obtained an average coverage of 35× across the opossum genome.

Read mapping and variant calling

Sequencing reads obtained from all 34 samples were mapped to the opossum reference genome MonDom5 (NCBI RefSeq GCF_000002295.2; Mikkelsen et al. 2007) using BWA-MEM v. 0.7.12-r1039 (Li 2013). Duplicate reads were identified and marked using Picard MarkDuplicates (v2.6.0; http://broadinstitute.github.io/picard/) to ensure accurate variant calling. Single nucleotide variants (SNVs) were called using the Genome Analysis Toolkit (GATK) version 4.1.2.0 (Van der Auwera et al. 2013) following recommended best practices. HaplotypeCaller was employed to generate genomic Variant Call Format files for each individual sample. This approach allows for the identification of variant sites along with genotype likelihoods. Subsequently, joint genotype calling across all samples was performed with GenotypeGVCFs, facilitating the comprehensive assessment of genetic variation within the opossum population. To ensure the integrity of the variant dataset, we applied stringent filtering criteria. Specifically, we implemented filters recommended by GATK, targeting single nucleotide polymorphisms (SNVs) with parameters set as follows: "QD < 2.0 || MQ < 40.0 || FS > 60.0 || SOR > 3.0 || MQRankSum < −12.5 || ReadPosRankSum < −8.0 and removed calls that failed. To assess the impact of inbreeding on mutation rates, we estimated inbreeding coefficients for non-offspring parents using VCFtools with the following command:

```
vcftools –vcf parents_only.recode.vcf –het –out parents_f
```

Identification of candidate mutations

We followed a framework outlined in our prior studies (Wang et al. 2020, 2022a,b) to detect autosomal *de novo* mutations from the set of called variants. Briefly, we restricted our search to a subset of sites identified as "Mendelian violations" within each trio by GATK. These are sites where both parents are homozygous for the reference allele, while the offspring is heterozygous for an alternate allele. To ensure the selection of high-confidence candidate mutation, we implemented a series of stringent filtering criteria:

- Read-depth filtering: We restricted candidate sites to those with a read-depth ranging between 20 and 60 for each individual within the trio. This step aims to mitigate sampling errors associated with inadequate coverage and addresses potential issues resulting from repetitive genomic regions.
- Genotype quality assessment: All individuals were subjected to stringent genotype quality thresholds (GQ > 70), ensuring reliable genotype calls across the cohort.
- Strand-specificity check: We required that candidate mutations were observed on both the forward and reverse strands in the offspring, as indicated by allelic depth metrics (ADF, ADR > 0).
- Parental validation: Mutations with more than 1 alternate read on either strand in nominally homozygous parents (AD = 0) were excluded to minimize genotyping errors and to ensure that selected variants were not present in the parental somatic tissue (i.e. the parent is actually heterozygous). The original BAM files were checked for such reads.
- Allelic balance filtering: Candidate mutations were further refined based on allelic balance criteria (Allelic Balance > 0.3), ensuring a balanced representation of alternate and reference alleles in the offspring.
- Pedigree exclusivity: Candidate mutations should exclusively be observed in samples directly descended from the parental lineage within each trio and not in any other individual in the population.

We applied these stringent filters to minimize the occurrence of false positive discoveries while ensuring the robustness of our mutation rate estimate, as demonstrated in Bergeron et al. (2022). Additionally, we monitored the transmission of de novo mutations across generations in our pedigrees, specifically for the 6 first-generation individuals who later became parents. Assessing the transmission of mutations to a third generation confirms that those mutations are inherited rather than being sequencing artifacts or somatic mutations.

Estimating the per-generation mutation rate

To estimate the per-generation mutation rate accurately, the counts of de novo mutations identified are divided by the total number of callable sites in each trio. Thus, we first defined the callable genome, the subset of the entire genome where reliable variant calls can be made with high confidence. We applied a methodology similar to the one used in previous studies (Besenbacher et al. 2019; Wang et al. 2020, 2022a,b). In these studies, a callable site is defined as one where sequencing depth and quality meet predefined criteria, so that the estimated probability of an identified candidate mutation is a true mutation is high. Defining a callable genome addresses false negatives by estimating the fraction of sites across the genome that can be accurately called in each trio. This approach allows us to correct for the false-negative rate without explicitly estimating it. The mutation rate calculation follows the equation:

$$\mu_i = \frac{N_{mut,i}}{2 \cdot \sum_x C_i(x)} \quad (1)$$

where μ_i is the per-site per-generation mutation rate for trio i, $N_{mut,i}$ is the number of de novo mutations identified in trio i, and $C_i(x)$ is the callability of site x in the trio. The denominator is multiplied by 2 because each parent contributes a haploid genome in which a mutation can be called in the offspring. This method assumes the independence of calling each individual in the trio correctly, which enables us to estimate $C_i(x)$ as the product of the probabilities of calling the child, father, and mother correctly as $C_c(x)$, $C_p(x)$, and $C_m(x)$, respectively, so that:

$$C_i(x) = C_c(x) \cdot C_p(x) \cdot C_m(x) \quad (2)$$

These probabilities are determined by applying the same set of stringent filters to high-confidence heterozygous calls from each trio. For heterozygous SNVs in the offspring, the callability $C_c(x)$ was estimated as the ratio of filtered heterozygous variants, $N_{het,filtered}$, to all heterozygous variants, $N_{het,all}$, where $N_{het,all}$, represents the number of variants in the offspring where 1 parent is homozygous for the reference, and the other parent is homozygous for the alternate allele. These numbers lead to an estimate of callability in the child:

$$C_c(x) = \frac{N_{het,filtered}}{N_{het,all}} \quad (3)$$

Similarly, parental callability, $C_p(x)$ and $C_m(x)$, was estimated by determining the proportion of remaining sites after applying stringent mutation filters. As implemented by Wang et al. (2020, 2022a,b) we evaluated the callability for each individual using a random sample of 250,000 sites across the genome that met the respective criteria for each trio.

Prediction of mutation rate from human data

To evaluate whether the observed mutation rates in opossums were consistent with expectations based on parental age, we utilized parameters estimated from human data (Jonsson et al. 2017). This model estimates the maternal and paternal contribution to the overall mutation rate in a trio, based on the age at conception of the parents.

Assuming a constant accumulation of mutations across the lifespan of the parents, we model the paternal and maternal contributions to a single offspring as follows:

$$\mu_p = \frac{6.05 + 1.51 \times a_p}{G} \quad (4)$$

$$\mu_m = \frac{3.61 + 0.37 \times a_m}{G} \quad (5)$$

where a_p and a_m represent the paternal and maternal ages at conception (in years), respectively, and G (=2.68×10^9 bp) is the callable genome size used by Jonsson et al. (2017). The constants in these equations (6.05, 1.51, 3.61, and 0.37) correspond to the intercepts and age coefficients derived from human mutation rate estimates (Jonsson et al. 2017). The total predicted per-generation mutation rate is then $(\mu_p + \mu_m)/2$ for a set of specified parental ages.

Assigning mutations to a parent of origin

To determine the parental origin of the de novo mutations identified in the 22 opossum trios, we utilized 2 tools: POOHA (https://github.com/besenbacher/POOHA [Maretty et al. 2017; Besenbacher et al. 2019; Bergeron et al. 2021]) and Unfazed (Belyeu et al. 2021). POOHA assigns the parent of origin by analyzing informative heterozygous single nucleotide polymorphisms (SNPs) that are located within the same sequencing read or read-pair as the DNM. We ran POOHA with a 1000 bp window around each DNM, using the variant call format (VCF) file for each trio and a BAM file for the child only.

In contrast, Unfazed implements a broader strategy, utilizing SNPs beyond the immediate vicinity of the DNM by linking them to parent-specific markers through haplotype reconstruction. Unfazed can extend the region of informative SNPs by chaining reads together using mutually overlapping heterozygous sites, allowing for assignment over longer distances than single read-pairs typically permit. This makes Unfazed particularly effective in cases where the parental source of the mutation is not defined directly by a nearby SNP. For each DNM, we provided Unfazed with the same 1000 bp window around each site as was used for POOHA, and the software used this window to search for informative sites.

To ensure accuracy, we cross-referenced the results from both tools, manually reviewing all assigned mutations using the Integrative Genomics Viewer (IGV) (Robinson et al. 2023).

Identification of postzygotic mutations

To infer the developmental timing of our set of de novo mutations, we implemented a read-based approach that involves physical linkage between candidate mutations and nearby heterozygous SNPs. While de novo mutations that occur in the parental germline are expected to be present in all offspring cells and on only 2 haplotypes, postzygotic mutations appear in only a subset of cells and are thus more likely to display haplotype mosaicism, i.e. more than 2 haplotypes (Supplementary Fig. 1; Sasani et al. 2019; Prentout et al. 2025). Our method allows us to determine

Table 1. Summary of mutation counts and mutation rate per trio.

Offspring ID	Parental age at conception (days)		Mutations	Phased mutations	Mutations of paternal origin	Mutations of maternal origin	Mean read depth	Callable genome size (Mb)	Mutation rate ($\times 10^{-8}$/bp)
	Paternal	Maternal							
646	286	167	6	6	3	3	35.3	2,201.29	0.136
648	190	167	16	11	10	1	35.0	2,153.665	0.371
649	270	247	9	8	5	3	36.0	2,220.75	0.203
652	298	345	6	4	2	2	36.0	2,215.335	0.135
653	398	445	13	8	5	3	36.3	2,256.59	0.288
654	484	531	20	17	11	6	36.0	2,071.365	0.483
657	393	252	7	7	2	5	32.7	2,059.23	0.17
659	168	180	14	12	6	6	35.0	2,222.33	0.315
660	293	305	15	10	7	3	35.0	2,275.71	0.33
661	372	384	7	5	3	2	34.7	2,225.295	0.157
664	237	197	10	7	7	0	34.3	2,178.465	0.23
665	362	322	15	9	3	6	34.7	2,264.995	0.331
666	472	432	12	9	7	2	34.7	2,216.325	0.271
669	328	146	9	9	8	1	34.7	2,206.54	0.204
671	278	209	10	7	7	0	34.7	2,200.54	0.227
672	390	321	10	4	3	1	34.3	2,198.17	0.227
673	504	435	10	8	4	4	34.3	2,174.16	0.23
676	198	293	12	10	5	5	36.3	2,200.665	0.273
677	323	418	20	15	12	3	35.7	2,167.075	0.461
650	286	167	6	5	3	2	35.3	2,186.755	0.137
662	393	252	6	5	3	2	33.0	2,071.105	0.145
674	N/A	N/A	9	8	6	2	35.0	2,153.915	0.209

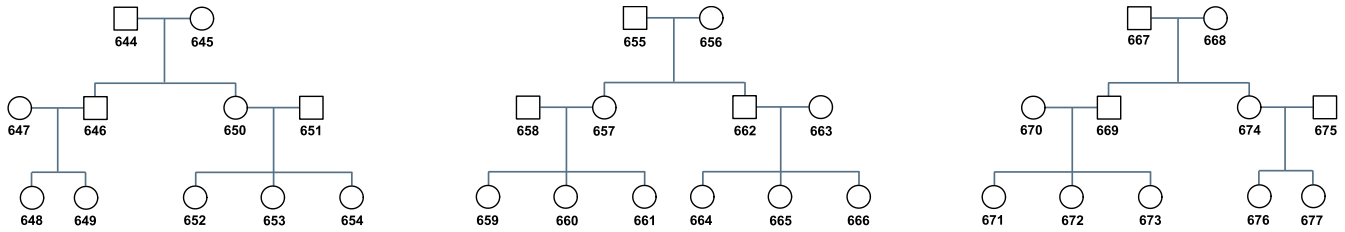

Fig. 1. Pedigree of sequenced gray short-tailed opossum individuals. Three extended pedigrees consisting of 34 individuals total were collected, which can be divided into 22 trios. Males are represented by squares and females by circles. IDs of all individuals correspond to those listed in Table 1.

whether mutations occurred postzygotically in the child of a trio more reliably than solely relying on allele balance.

For each candidate mutation, we looked for nearby heterozygous SNPs in the child to build a haplotype. We extracted sequencing reads from the corresponding BAM file whose coordinates span both the mutation and at least 1 nearby SNP, recording the observed bases at both sites. For the focal mutation and a nearby SNP, we counted occurrences of the 4 possible haplotype combinations given reference and alternate alleles. If multiple nearby SNPs were available, the SNP with the highest number of informative reads (i.e. covering both sites) was used.

Mutations in regions with exactly 2 haplotypes in the child were classified as arising in the germline of the parent; i.e. the mutation was observed exclusively on 1 parental haplotype (Supplementary Fig. 1a). In contrast, mutations arising postzygotically will create 3 haplotypes in the child, with both the mutant allele and the reference allele associated with a single parental allele at the neighboring SNP (Supplementary Fig. 1b). We required at least 2 reads supporting the third haplotype to label a mutation as postzygotic. Mutations with insufficient data were considered undetermined.

In order to estimate the false positive rate of this approach, we looked at the rate at which a third haplotype was detected in the child during the routine transmission of SNP variants. Using the same criteria described above, we examined the haplotypes surrounding a random set of SNPs that were homozygous for different alleles in the parents and heterozygous in the offspring. We considered the detection of a third haplotype in the child for any reason to count toward the false positive rate. This process was repeated 20 times on independent sets of 1000 SNPs.

Results

Estimating the mutation rate from opossum pedigrees

The gray short-tailed opossum becomes sexually mature at 5 to 6 mo and can live for 36 to 42 mo in captivity (Macrini 2004). All individuals in this study reproduced before they were 17 mo old (Table 1). In total, we sequenced the genomes of 34 individuals from 3, three-generation extended pedigrees (Fig. 1) of captive opossums maintained at Texas A&M University. The entire F_2 generation consists of females; however, this does not affect our analysis, as we are not specifically investigating somatic mutations in the offspring. Genomic DNA was isolated and sequenced on Illumina Hi-Seq X instruments, producing 150 bp paired-end reads to an average of 35× coverage (min: 31×, max: 38×) (Table 1; Materials and Methods). The pedigrees can be separated into 22 trios, and we estimated the mutation rate for each trio independently. Filtering strategies were used to retain only sites with optimal quality and coverage, which resulted in an average

callable genome of 2.2 Gb per trio (Table 1). We also estimated inbreeding coefficients for the parents in our pedigrees, which ranged from 0.32 to 0.86, with a mean of 0.544 (95% CI: 0.414 to 0.674).

After stringent filtering to reduce the incidence of false positives, we identified a total of 242 single nucleotide de novo mutations across the 22 trios (Supplementary Table 1), 4 of which were multinucleotide mutations (Schrider et al. 2011). The number of DNMs identified in each trio ranged from as low as 6 to as high as 20 (Table 1). Among our candidate mutations, 5 genomic positions were shared between 4 pairs of siblings, implying that these mutations were mosaic in the germline of a parent. This finding is consistent with previous reports (Sasani et al. 2019), which estimate that around 3% of de novo mutations arise as mosaic mutations in the parental germ cells. We also tracked the transmission of DNMs across generations within our pedigrees for the 6 individuals in the first generation that went on to become parents. A total of 43 detected mutations could be tracked, of which 45.6%, on average, were transmitted to offspring in the next generation; this transmission rate is not different from the 50% expectation ($P = 0.4$, Exact test). These results help to strengthen the inference that these are true DNMs.

Considering all trios and DNMs, we estimated an average mutation rate of 0.252×10^{-8} per base pair per generation (95% confidence interval [CI]: 0.208 to 0.295×10^{-8}) for parents at an average age of 313 d across sexes (♂:330 d ♀:296 d). In humans, per-generation estimates are 1.29×10^{-8} per base pair with an average parental age of 30.1 yr (Jonsson et al. 2017). Using regression estimates based on human data (Methods) and applying them to opossums after adjusting for differences in the sizes of the callable genomes, we predict an average mutation rate of 0.211×10^{-8} per base pair per generation (95% CI: 0.207 to 0.215×10^{-8}) for humans that have children at the same age as the average opossum. This prediction closely matches our observed mutation rate (and overlaps its CI) (Supplementary Fig. 2), further highlighting the significant role of parental age in determining mutation rates across different species.

Parental contributions to the mutation rate

We used 2 tools, POOHA (Maretty et al. 2017; Bergeron et al. 2021) and Unfazed (Belyeu et al. 2021), to determine the parent of origin for DNMs across the 22 trios (sometimes this process is called "phasing"). POOHA assigned 189 of the 242 mutations, while Unfazed assigned only 168 mutations. Although Unfazed also successfully assigned 2 mutations that POOHA could not, both programs assigned the same parent of origin for all shared mutations, with no disagreements. To ensure that all the mutations were assigned correctly, we manually inspected all assigned mutations in IGV. We found 2 errors from Unfazed, whereas POOHA assigned 5 mutations incorrectly. Therefore, we excluded these cases from the set of mutations with determined parental origin in our subsequent analyses. However, despite these minor discrepancies, both tools provided reliable results overall. These steps resulted in 184 mutations (76.0%) assigned by POOHA, and 166 mutations (68.6%) assigned by Unfazed. Based on the larger number of mutations assigned to 1 parent or the other, we proceeded with the POOHA results for the remainder of our analyses.

Of the 184 assigned mutations, 122 (66.3%) were of paternal origin and 62 (33.7%) were of maternal origin (Table 1). This indicates a strong male mutation bias, with nearly two-thirds of mutations being paternal. However, this bias is slightly lower than the 80.4% reported in humans (Jonsson et al. 2017) and the 75% to80% observed in other mammals (Tatsumoto et al. 2017; Wang et al. 2022a,b). We find that our observed fraction of paternal mutations

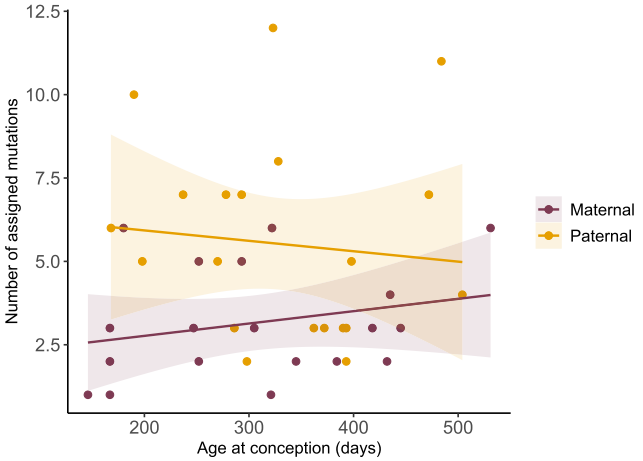

Fig. 2. Parental age at conception does not correlate with offspring mutation count. Each dot represents the number of mutations found in a single offspring, color-coded by parent of origin. The solid lines are the best-fit regression for the relationship between parental age and mutation count, with shaded areas representing the 95% confidence intervals.

is significantly lower than that among humans ($\chi^2 = 21.91$; $P < 0.00001$) and significantly lower than an expectation of 75% ($\chi^2 = 7.42$; $P = 0.006$).

In contrast to what has been observed in other mammals (Venn et al. 2014; Rahbari et al. 2016; Thomas et al. 2018; Sasani et al. 2019; Wang et al. 2020,2022b), we did not find a parental age effect on the assigned mutations (Fig. 2). Neither paternal age nor maternal age had a significant effect on either the total number of mutations or the number of sex-specific mutations (i.e. an effect of maternal age on maternally transmitted mutations). This remains the case when using up-scaled counts that account for variation in the fraction of mutations assigned to a parent of origin (Supplementary Fig. 3).

One potential explanation for the lack of a parental age effect could be the small age-range of parents in our study: the youngest parent differs by only 1 yr from the oldest (Table 1; Fig. 2). To determine whether this narrow age range could be the reason why we did not observe a trend in opossums, we evaluated the same age-range restrictions in a human dataset. Using the data from Jonsson et al. (2017), we analyzed pairs of data points 1 yr apart (the human data only has resolution to the nearest year). We identified 44 such pairs, where consecutive ages were available in the dataset, and then asked whether there was a significant effect of paternal age on the number of paternally transmitted mutations. Only 7 out of 44 pairs showed a significant paternal age effect (Supplementary Fig. 4), even though there is a strong effect when analyzing all the data together (see figure 1 in Jonsson et al. 2017). These results suggest that an age effect in opossums as strong as the 1 in humans might not be detected due to the narrow age range among parents in our opossum sample.

The developmental origin of mutations

Mutations that occur during the first few cycles of cell division in a developing zygote may reach high variant allele frequencies, making them difficult to distinguish from mutations occurring in the parental germline. To investigate the developmental timing of the mutations we identified, we applied a read-based approach that uses physical linkage with nearby heterozygous SNPs. Out

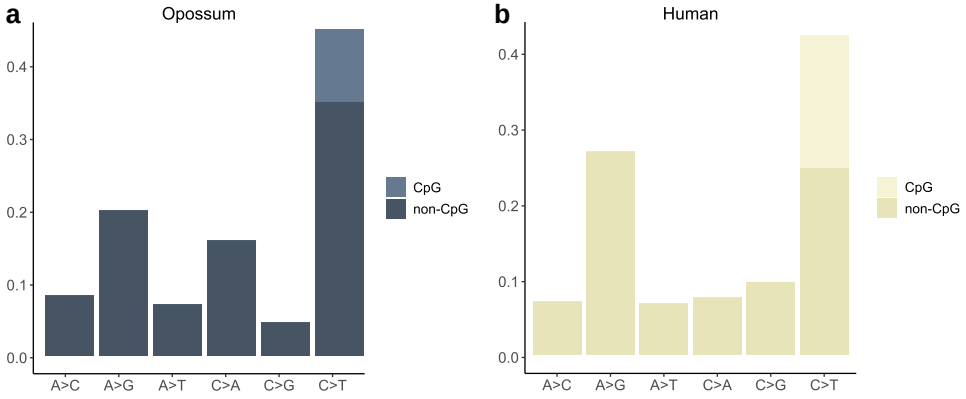

Fig. 3. Opossum mutation spectrum compared to humans. a) The proportion of each mutation class observed among gray short-tailed opossum trios, including their reverse complements. Mutation classes are categorized based on nucleotide changes (e.g. C→T, G→A). b) The same mutation spectrum for human trios, highlighting similarities and differences in mutation patterns between the 2 species. Data on humans from Jonsson et al. (2017).

of 242 mutations, we were able to confidently determine the timing for 148. Of these, 127 mutations were supported by reads forming exactly 2 distinct haplotypes, suggesting that they arose in the parental germline. In contrast, 21 mutations (14.2%) exhibited 3 haplotypes, indicative of postzygotic mosaicism (see Materials and Methods). This proportion of postzygotic mutations is comparable to estimates reported in some other species, including human (Porubsky et al. 2025) and zebra finch (Prentout et al. 2025), but not as large as the observed in other organisms such as cattle (Harland et al. 2017) or mice (Lindsay et al. 2019).

To test the accuracy of the 3-haplotype method, we further evaluated a set of control SNPs for which only 2 haplotypes should be present. Applying our approach to these SNPs, we found that an average of 1.62% of them had more than 2 haplotypes, revealing a false positive rate for our approach. When applying a more permissive classification strategy (as used in some previous studies, where a single read is sufficient to support a third haplotype), the misclassification rate increased to 6.96%. These results suggest that our more stringent approach improves classification accuracy by reducing false positives caused by sequencing errors, low-quality alignments, or mapping artifacts. They also suggest that the true fraction of postzygotic mutations in our study is closer to 12.5%.

Mutation spectrum in opossum

We examined the mutation spectrum from all identified mutations, classifying each into 1 of 6 mutation types (Fig. 3a). While the frequency of some mutation classes closely resembled those found in humans (Fig. 3b), the overall mutation spectrum was significantly different between the 2 species ($\chi^2 = 32.56$; $P = 0.000005$). The transition-to-transversion (Ts/Tv) ratio in opossum was 1.81, comparable to the ratio observed in other mammals, and not significantly different than the human ratio of 2.22 (Jonsson et al. 2017) ($\chi^2 = 2.07$;$P = 0.15$).

C > T transitions were the most common type of mutation, observed in 45% (108 out of 242) of all changes, similar to the 42.1% reported in humans (Jonsson et al. 2017). However, only 9.9% of the total number of mutations arose at CpG sites, lower than the 20.8% of all mutations estimated in humans (Besenbacher et al. 2015) or 21% in cats (Wang et al. 2022b). One possible explanation for the lower fraction of CpG mutations is that the CpG content in the opossum genome is low. At 0.44%, the CpG content in the opossum genome assembly (MonDom5) is less than half of what it is found to be in the human genome (hg38), 1.03%. After accounting for this difference in CpG content, the discrepancy in the fraction of mutations arising at CpG sites is no longer significant (χ^2=1.54, $P = 0.214$), suggesting that the lower CpG content is sufficient to explain the reduced proportion of CpG > TpG mutations in opossums, rather than any differences in CpG-specific mutation rates. Though CpG sites are a smaller fraction of the opossum genome, we estimate the mutation rate at these sites to be 5.58×10^{-8} per bp per generation, 22.5× higher than the overall rate, and similar to the multiple for CpG sites relative to the overall rate in both humans and cats (21.4× and 21.7×, respectively; Besenbacher et al. 2015; Wang et al. 2022b).

To ask whether the difference in mutation spectrum between humans and opossums is solely due to the lower number of CpG mutations in opossums, we reanalyzed the data excluding CpG sites. Even after removing this group, the mutation spectrum remained significantly different between the 2 species ($\chi^2 = 36.96$; $P = 0.000001$). To further explore whether the mutation spectrum observed in opossums could be explained by their age at reproduction, we applied a method previously described in Wang et al. (2022b). This approach, originally developed to model the mutation patterns in cats relative to humans, uses Poisson regression to predict the mutation spectrum based on parental age. Counts for each mutation class are independently modeled as a function of the father's and mother's age at conception using a Poisson regression. When applied to our opossum mutation data, the predicted mutation spectrum still differed substantially from the one we observed ($\chi^2 = 12.41$; $P = 0.03$) (Supplementary Fig. 5). However, this difference was smaller compared to the observed human mutation spectrum, suggesting that while some aspects of the mutation spectrum have evolved between humans and opossums, accounting for dinucleotide content and parental age at conception reduces the discrepancy.

To evaluate variation in the types of mutations inherited from mothers versus fathers, we analyzed the sex-specific mutation spectrum (Supplementary Fig. 6). We observed that the Ts/Tv ratio for males was 2.30, while for females it was lower at 1.38. Similarly, C > T transitions, the most common mutation type, accounted for 48.18% of paternal mutations and 35.5% of maternal mutations. The occurrence of C > T transitions at CpG sites was also less frequent in maternal mutations, at 4.84%, compared to 10.66% in paternal mutations. While none of these differences reached statistical significance ($P > 0.05$), they suggest potential sex-specific trends in the mutation spectrum that merit further exploration.

Discussion

The results presented here highlight important aspects of the evolution of mutation rates and sex-biased mutation. We identified a total of 242 germline mutations in 22 opossum trios, which enabled us to estimate an average mutation rate of 0.252×10^{-8} per base pair per generation for parents at an average age of 313 d. This estimate of the mutation rate in opossums is among the lowest values observed so far in mammals (Bergeron et al. 2023). A previous estimate derived from a single trio of the same species had a value of 0.46×10^{-8} (Bergeron et al. 2023), but the parents in that trio had an average age of 1.5 yr, which could explain the higher rate observed. While our dataset improves upon earlier work by including more families and a wider range of parental ages, it is important to recognize that the individuals sampled still represent a limited window of the species' reproductive lifespan, typical of what is observed in captive colonies. This restricted age range may limit our ability to detect subtle age-related increases in mutation rates, and should be considered when interpreting the apparent absence of a parental age effect in our study.

The age at conception appears to be the primary determinant of both the mutation rate per generation (Thomas et al. 2018; Bergeron et al. 2023) and the mutation spectrum (Wang et al. 2022b; Beichman et al. 2023) among mammals. These relationships are reasonable when we consider that both the number of mutations and the spectrum of mutations change with the age of parents within species (Jonsson et al. 2017). Given the association between age at conception and mutation rate, one can ask whether the rate we observe in opossums is consistent with expectations based on parental age. Using Poisson regression estimates for the number of mutations expected from male and female parents in humans (Jonsson et al. 2017), we predicted the mutation rate in opossums based on the observed parental ages in our trios. The model, adjusted for differences between the callable genome sizes in humans and opossums, predicted a mutation rate of 0.211×10^{-8} per base pair per generation. This estimate closely matches the observed mutation rate of 0.252×10^{-8}, with no statistically significant difference based on a comparison with the 95% CI of the estimated rate. While this similarity is compelling, it is important to consider the assumptions underlying the human model and its applicability to a marsupial species with distinct reproductive biology and life-history traits. The human mutation model assumes a relatively constant accumulation of mutations throughout reproductive life, with a fixed number of mutations present in the germline before reproduction. However, early stages of gametogenesis may differ significantly across species. In humans, for instance, spermatogonia remain dormant throughout childhood and only gradually and modestly activate over the first ~5 yr of life (Wu et al. 2009). If this process influences the accumulation of mutations, then extrapolating human rates backward to an age at which opossums reproduce may not accurately capture how mutations arise in germ cells of early-reproducing species. Moreover, while the close agreement between the predicted and observed mutation rates in opossums suggests evolutionary conservation of mutation rates across mammals, the mutation spectrum analysis also shows that there are key differences in mutational mechanisms. The composition and relative proportions of different mutation types in opossums differ from those seen in humans, indicating that similar rates do not necessarily imply completely shared mechanisms. Another limitation of the model is that it assumes that parental age is the main determinant of the mutation rates. However, recent findings have shown that the relationship between parental age and the mutation rate may vary significantly, even within species (Zhang et al. 2024). Future work comparing germline mutation spectra across species with diverse life histories will be essential to disentangle how early-life processes shape both the rate and spectrum of mutations and whether shared evolutionary constraints influence these patterns.

While opossums show one of the lowest mutation rates among mammals so far, Bergeron et al. (2023) found similarly low mutation rates in multiple species, though most of these estimates were based on a single trio, which can lead to limitations in the overall mutation rate estimate. For example, the cat mutation rate was estimated at 0.35×10^{-8} from 1 trio, whereas Wang et al. (2022b), using 11 trios, reported a higher rate of 0.86×10^{-8}. Differences among studies can also arise from differences in the exact ages of the parents included, even when the underlying parameters of mutation accumulation are the same (e.g. compare rhesus macaque rates between Wang et al. (2020) and Bergeron et al. (2021)). Although opossum mutation rates are low, they are not the species with the youngest parents tested: laboratory mice are (Lindsay et al. 2019; Lopez-Cortegano et al. 2024). Previous work on commonly used inbred strains of mice have reported mutation rates ranging from 0.39×10^{-8} per base pair per generation for individuals with an average parental age of 171.5 d (Lindsay et al. 2019) to 0.67×10^{-8} per base pair per generation for parents with an average age of approximately 150 d (Lopez-Cortegano et al. 2024).These estimates are higher than predicted by our model given the age of the parents, making laboratory mice an outlier. To evaluate the potential impact of inbreeding on our dataset, we estimated inbreeding coefficients for the parents in our pedigrees and found moderate to high values (mean = 0.544, 95% CI: 0.414 to 0.674). Despite this, our estimated mutation rates were not outliers, suggesting that inbreeding alone does not always lead to elevated mutation rates. Although Bergeron et al. (2023) found higher mutation rates per year in domesticated and laboratory species, this difference went away after controlling for the shorter average generation times in these animals. However, it is also important to note that the mutation rates observed in captive opossums may not be directly comparable to those of wild populations. Further studies will be needed to help to clarify the extent to which laboratory adaptation could influence de novo mutation rates.

Our analysis of mutations transmitted by each parent revealed a marked male mutation bias, even among very young parents, with fathers passing on two-thirds of the mutations observed in the offspring. Pedigree-based studies, reliant on observing transmitted germline mutations among individuals that have reproduced, face limitations in assessing potential sex biases before puberty. Consequently, our understanding of male bias is limited by the age of reproductive maturity at which individuals can contribute genetic material to the next generation. The gray short-tailed opossum, with its early onset of puberty compared to many mammals, provides an ideal organism to study male bias.

Replication errors have long been thought to be a primary source of germline mutations (Haldane 1946), especially as the male germline undergoes many more replications postpuberty. If replication were the primary driver, one might expect a much weaker bias among younger fathers, as males and females will have undergone a similar number of replications before producing their gametes. Although we do observe a slightly reduced male bias in opossums—most mammals inherit three-quarters of mutations through the male parent (de Manuel et al. 2022)—the reduced male bias observed in opossums may reflect a combination of biological, evolutionary, and ecological factors. The

early reproductive onset in opossums likely limits the time available for differential mutation accumulation between males and females. If replication is a major driver of male bias, male opossums may simply not have had as much time to accumulate mutations relative to females. This timing could reduce the extent of male bias that might otherwise become more evident with age, as seen in other mammals.

Additionally, the evolutionary divergence between marsupials and eutherian mammals may have resulted in distinct mechanisms, underlying differences between the sexes. The long evolutionary separation between these lineages could have led to unique changes in gametogenesis or germline maintenance among marsupials, producing sex-specific mutation patterns distinct from those of eutherian mammals. Environmental factors, such as differences in mutagen exposure between sexes, may also shape the observed bias. Male opossums, like other mammals, may experience greater environmental stresses due to behavioral or ecological roles that increase contact with mutagens. However, in a species with shorter generational cycles, the impact of such exposure may be constrained by time, and both sexes of our study animals were raised with essentially the same exposure to environmental influences.

Despite the reduced male bias in opossums, our results are still quite similar to those in other species with much longer generation times. Some models suggest that younger parents should exhibit lower male bias (Gao et al. 2019), though the data from humans do not show such a pattern. This study, therefore, contributes to a growing body of evidence that additional factors beyond DNA replication play a substantial role in shaping the observed paternal bias (Gao et al. 2019; de Manuel et al. 2022), including possible differences in DNA repair mechanisms, environmental exposures, or selective pressures acting on the male and female germlines (Hahn et al. 2023). Of course, the slightly depressed degree of male-bias in opossums could be explained by a relative lack of male germline replication given the short time between puberty and reproduction, but it would only then explain a small fraction of this bias.

We found a significantly different overall mutation spectrum in opossums compared to that reported for humans, consistent with lineage-specific changes in mutational machinery. Among the differences, a notable one is observed in the fraction of C > T mutations occurring at CpG sites, which is lower in opossums compared to humans. An important factor contributing to the reduced proportion of C > T mutations at CpG sites in opossums may be their relatively low CpG content compared to other mammalian species. Specifically, opossums harbor less than half the CpG content found in humans (Mikkelsen et al. 2007), suggesting that CpG depletion could play a significant role in shaping the mutation spectrum. This highlights the significance of genome composition and context in shaping mutation patterns across species. Thus, while variation in generation time may provide insights into certain aspects of mutational spectra (e.g. Jonsson et al. 2017; Wang et al. 2023), the low CpG content in opossums underscores the importance of considering species-specific genomic features when interpreting mutation patterns.

In conclusion, further research into the molecular processes underlying germline mutations, particularly during early stages of reproductive maturity, will be essential for unraveling the intricate interplay of factors shaping mutation patterns in mammals. By using a model short-lived marsupial, our research provides valuable insights into mutation rates, male bias, and evolutionary implications, both within marsupials and across mammals. The findings presented here therefore contribute to a broader understanding of the evolutionary processes underlying key evolutionary traits.

Data availability

The raw sequencing reads are available from the NCBI SRA database under accession number PRJNA1139788, https://www.ncbi.nlm.nih.gov/bioproject/?term = PRJNA1139788. The corresponding VCF files are available at https://doi.org/10.5281/zenodo.15285301.

Supplemental material available at GENETICS online.

Acknowledgments

We wish to thank Donna Muzny, Harshavardhan Doddapaneni, Richard Gibbs and their teams at the Human Genome Sequencing Center, Baylor College of Medicine for expert genome sequencing in support of this study. John VandeBerg, Michael Nachman, and 3 reviewers all provided helpful comments on the manuscript.

Funding

National Institutes of Health grant R01-HD107120.

Author contributions

Y.P.-G., R.J.W., J.R., and M.W.H. conceived analyses; Y.P.-G., R.A.H., and R.J.W., performed analyses; P.B.S. provided the opossum samples; M.R. oversaw extraction of DNA and the production of sequencing libraries; M.W.H. and J.R. supervised sequencing and analysis.

Conflicts of interest. None declared.

Literature cited

Armstrong EE *et al.* 2025. Parameterizing Pantherinae: *de novo* mutation rate estimates from *Panthera* and *Neofelis* pedigrees. Genome Biol Evol. 17:evaf060. https://doi.org/10.1093/gbe/evaf060.

Beichman AC *et al.* 2023. Evolution of the mutation spectrum across a mammalian phylogeny. Mol Biol Evol. 40:msad213. https://doi.org/10.1093/molbev/msad213.

Belyeu JR, Sasani TA, Pedersen BS, Quinlan AR. 2021. Unfazed: parent-of-origin detection for large and small *de novo* variants. Bioinformatics. 37:4860–4861. https://doi.org/10.1093/bioinformatics/btab454.

Bergeron LA *et al.* 2021. The germline mutational process in rhesus macaque and its implications for phylogenetic dating. Gigascience. 10:giab029. https://doi.org/10.1093/gigascience/giab029.

Bergeron LA *et al.* 2022. The mutationathon highlights the importance of reaching standardization in estimates of pedigree-based germline mutation rates. Elife. 11:e73577. https://doi.org/10.7554/eLife.73577.

Bergeron LA *et al.* 2023. Evolution of the germline mutation rate across vertebrates. Nature. 615:285–291. https://doi.org/10.1038/s41586-023-05752-y.

Besenbacher S *et al.* 2015. Novel variation and *de novo* mutation rates in population-wide *de novo* assembled Danish trios. Nat Commun. 6:5969. https://doi.org/10.1038/ncomms6969.

Besenbacher S, Hvilsom C, Marques-Bonet T, Mailund T, Schierup MH. 2019. Direct estimation of mutations in great apes reconciles phylogenetic dating. Nat Ecol Evol. 3:286–292. https://doi.org/10.1038/s41559-018-0778-x.

Crow JF. 2000. The origins, patterns and implications of human spontaneous mutation. Nat Rev Genet. 1:40–47. https://doi.org/10.1038/35049558.

de Manuel M, Wu FL, Przeworski M. 2022. A paternal bias in germline mutation is widespread in amniotes and can arise independently of cell division numbers. Elife. 11:e80008. https://doi.org/10.7554/eLife.80008.

Drost JB, Lee WR. 1995. Biological basis of germline mutation: comparisons of spontaneous germline mutation rates among Drosophila, mouse, and human. Environ Mol Mutagen. 25: 48–64. https://doi.org/10.1002/em.2850250609.

Gao Z *et al.* 2019. Overlooked roles of DNA damage and maternal age in generating human germline mutations. Proc Natl Acad Sci U S A. 116:9491–9500. https://doi.org/10.1073/pnas.1901259116.

Goldmann JM *et al.* 2016. Parent-of-origin-specific signatures of *de novo* mutations. Nat Genet. 48:935–939. https://doi.org/10.1038/ng.3597.

Hahn MW, Pena-Garcia Y, Wang RJ. 2023. The 'faulty male' hypothesis for sex-biased mutation and disease. Curr Biol. 33: R1166–R1172. https://doi.org/10.1016/j.cub.2023.09.028.

Haldane JBS. 1935. The rate of spontaneous mutation of a human gene. J Genet. 31:317–326. https://doi.org/10.1007/BF02982403.

Haldane JBS. 1946. The mutation rate of the gene for haemophilia, and its segregation ratios in males and females. Ann Eugen. 13: 262–271. https://doi.org/10.1111/j.1469-1809.1946.tb02367.x.

Harland C *et al.* Frequency of mosaicism points towards mutation-prone early cleavage cell divisions in cattle. bioRxiv 079863. https://doi.org/10.1101/079863, 29 June 2017, preprint: not peer reviewed.

Jonsson H *et al.* 2017. Parental influence on human germline *de novo* mutations in 1,548 trios from Iceland. Nature. 549:519–522. https://doi.org/10.1038/nature24018.

Koch EM *et al.* 2019. *De novo* mutation rate estimation in wolves of known pedigree. Mol Biol Evol. 36:2536–2547. https://doi.org/10.1093/molbev/msz159.

Kong A *et al.* 2012. Rate of *de novo* mutations and the importance of father's age to disease risk. Nature. 488:471–475. https://doi.org/10.1038/nature11396.

Li H. 2013. Aligning sequence reads, clone sequences and assembly contigs with BWA-MEM [preprint], arXiv:1303.3997v2. URL preferably DOI:https://doi.org/10.48550/arXiv.1303.3997.

Lindsay SJ, Rahbari R, Kaplanis J, Keane T, Hurles ME. 2019. Similarities and differences in patterns of germline mutation between mice and humans. Nat Commun. 10:4053. https://doi.org/10.1038/s41467-019-12023-w.

Lopez-Cortegano E *et al.* 2024. Variation in the spectrum of new mutations among inbred strains of mice. Mol Biol Evol. 41:msae163. https://doi.org/10.1093/molbev/msae163.

Luo ZX, Yuan CX, Meng QJ, Ji Q. 2011. A jurassic eutherian mammal and divergence of marsupials and placentals. Nature. 476: 442–445. https://doi.org/10.1038/nature10291.

Macrini TE. 2004. Monodelphis domestica. Mammalian Species. 760: 1–8. https://doi.org/10.1644/760.

Maretty L *et al.* 2017. Sequencing and *de novo* assembly of 150 genomes from Denmark as a population reference. Nature. 548: 87–91. https://doi.org/10.1038/nature23264.

Mikkelsen TS *et al.* 2007. Genome of the marsupial *Monodelphis domestica* reveals innovation in non-coding sequences. Nature. 447: 167–177. https://doi.org/10.1038/nature05805.

Nachman MW, Crowell SL. 2000. Estimate of the mutation rate per nucleotide in humans. Genetics. 156:297–304. https://doi.org/10.1093/genetics/156.1.297.

Porubsky D *et al.* 2025. Human *de novo* mutation rates from a four-generation pedigree reference. Nature. 643:427–436. https://doi.org/10.1038/s41586-025-08922-2.

Prentout D *et al.* Mutation and recombination parameters in zebra finch are similar to those in mammals. bioRxiv 611523. https://doi.org/10.1101/2024.09.05.611523, 17 February 2025, preprint: not peer reviewed.

Rahbari R *et al.* 2016. Timing, rates and spectra of human germline mutation. Nat Genet. 48:126–133. https://doi.org/10.1038/ng.3469.

Robinson JT, Thorvaldsdottir H, Turner D, Mesirov JP. 2023. Igv.js: an embeddable JavaScript implementation of the integrative genomics viewer (IGV). Bioinformatics. 39:btac830. https://doi.org/10.1093/bioinformatics/btac830.

Sasani TA *et al.* 2019. Large, three-generation human families reveal post-zygotic mosaicism and variability in germline mutation accumulation. Elife. 8:e46922. https://doi.org/10.7554/eLife.46922.

Schrider DR, Hourmozdi JN, Hahn MW. 2011. Pervasive multinucleotide mutational events in eukaryotes. Curr Biol. 21:1051–1054. https://doi.org/10.1016/j.cub.2011.05.013.

Silva JC, Kondrashov AS. 2002. Patterns in spontaneous mutation revealed by human-baboon sequence comparison. Trends Genet. 18:544–547. https://doi.org/10.1016/s0168-9525(02)02757-9.

Suarez-Menendez M *et al.* 2023. Wild pedigrees inform mutation rates and historic abundance in baleen whales. Science. 381: 990–995. https://doi.org/10.1126/science.adf2160.

Tatsumoto S *et al.* 2017. Direct estimation of *de novo* mutation rates in a chimpanzee parent-offspring trio by ultra-deep whole genome sequencing. Sci Rep. 7:13561. https://doi.org/10.1038/s41598-017-13919-7.

Thomas GWC *et al.* 2018. Reproductive longevity predicts mutation rates in primates. Curr Biol. 28:3193–3197.e5. https://doi.org/10.1016/j.cub.2018.08.050.

VandeBerg JL, Robinson ES. 1997. The laboratory opossum (*Monodelphis Domestica*) in laboratory research. ILAR J. 38:4–12. https://doi.org/10.1093/ilar.38.1.4.

Van der Auwera GA *et al.* 2013. From FastQ data to high confidence variant calls: the genome analysis toolkit best practices pipeline. Curr Protoc Bioinformatics. 43:11.10.11–11.10.33. https://doi.org/10.1002/0471250953.bi1110s43.

Venn O *et al.* 2014. Strong male bias drives germline mutation in chimpanzees. Science. 344:1272–1275. https://doi.org/10.1126/science.344.6189.1272.

Wang RJ *et al.* 2020. Paternal age in rhesus macaques is positively associated with germline mutation accumulation but not with measures of offspring sociability. Genome Res. 30:826–834. https://doi.org/10.1101/gr.255174.119.

Wang RJ *et al.* 2022a. Examining the effects of hibernation on germline mutation rates in grizzly bears. Genome Biol Evol. 14: evac148. https://doi.org/10.1093/gbe/evac148.

Wang RJ *et al.* 2022b. *De novo* mutations in domestic cat are consistent with an effect of reproductive longevity on both the rate and spectrum of mutations. Mol Biol Evol. 39:msac147. https://doi.org/10.1093/molbev/msac147.

Wang RJ, Al-Saffar SI, Rogers J, Hahn MW. 2023. Human generation times across the past 250,000 years. Sci Adv. 9:eabm7047. https://doi.org/10.1126/sciadv.abm7047.

Wilson Sayres MA, Makova KD. 2011. Genome analyses substantiate male mutation bias in many species. Bioessays. 33:938–945. https://doi.org/10.1002/bies.201100091.

Wong WS *et al.* 2016. New observations on maternal age effect on germline *de novo* mutations. Nat Commun. 7:10486. https://doi.org/10.1038/ncomms10486.

Wu FL *et al.* 2020. A comparison of humans and baboons suggests germline mutation rates do not track cell divisions.

PLoS Biol. 18:e3000838. https://doi.org/10.1371/journal.pbio.3000838.

Wu X *et al.* 2009. Prepubertal human spermatogonia and mouse gonocytes share conserved gene expression of germline stem cell regulatory molecules. Proc Natl Acad Sci U S A. 106:21672–21677. https://doi.org/10.1073/pnas.0912432106.

Xiong X *et al.* 2022. Genetic and genomic architecture in eight strains of the laboratory opossum *Monodelphis domestica*. G3 (Bethesda). 12:jkab389. https://doi.org/10.1093/g3journal/jkab389.

Yoder AD, Tiley GP. 2021. The challenge and promise of estimating the *de novo* mutation rate from whole-genome comparisons among closely related individuals. Mol Ecol. 30:6087–6100. https://doi.org/10.1111/mec.16007.

Zhang S-J *et al.* 2024 Jun 5. Determinants of *de novo* mutations in extended pedigrees of 43 dog breeds [preprint]. bioRxiv 596747. https://doi.org/10.1101/2024.06.04.596747.

Zhu L, Beichman A, Harris K. 2025. Population size interacts with reproductive longevity to shape the germline mutation rate. Proc Natl Acad Sci U S A. 122:e2423311122. https://doi.org/10.1073/pnas.2423311122.

Editor: M. Nachman