Selection against mitochondrial mutations occurs across life stages in *Drosophila*

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Abstract

The accumulation of heteroplasmy -or genetic variation of mitochondrial DNA within the body- is associated with aging, neurodegenerative disease, cancer, and muscle degeneration disorders. Somatic mitochondrial mutations accumulate through the lifespan of an organism, and it is important to understand the frequencies, rates, and sources of natural selection that drive the accumulation and abundance of such mutations. *Drosophila simulans* is a potentially valuable model organism for studies of mitochondrial mutation load because they have short generation times, can be sampled at many life stages, and have a well annotated genome. Using an ultra-accurate sequencing technique capable of detecting low-frequency variation within a single tissue sample, I identified very low-frequency mutations in *D. simulans* individuals with high accuracy. By analyzing multiple individuals across different life stages, we compare mitochondrial mutational loads and evaluate the accumulation of heteroplasmy through the lifecycle of the flies. We quantified mutational impact on mitochondrially encoded genes known to be crucial for the production of ATP, revealing selective pressure on missense mutations across life stages. The discovery of selection against missense mutations has implications for our broader understanding of heteroplasmy and can provide insights into the diseases and disorders that it contributes to.

Acknowledgments

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1 Background

1.1 Mitochondrial DNA

In eukaryotic cells, genetic material is encoded in both the nuclear and the mitochondrial genomes. The mitochondrion is an organelle responsible for the production of energy in cells (Figure 1). Mitochondria have a relatively high mutation rate relative to the nuclear genome, and because there can be up to 2000 mitochondria in each cell, heteroplasmy, or genetic variation, among mitochondria, can accumulate within each cell and cell lineage.[1–3] If only a few mitochondria are disabled by mutation, the cell may survive, but if a large percentage are heavily mutated (high heteroplasmy), it can compromise cellular function.[3] The passing on of harmful mutations from one generation to the next is mitigated by the process of gamete production, as it limits the transmission of genomes from the primordial germ cell to the oocyte, meaning only the mitochondria with the lowest mutational burned are passed to the next generation.[4]

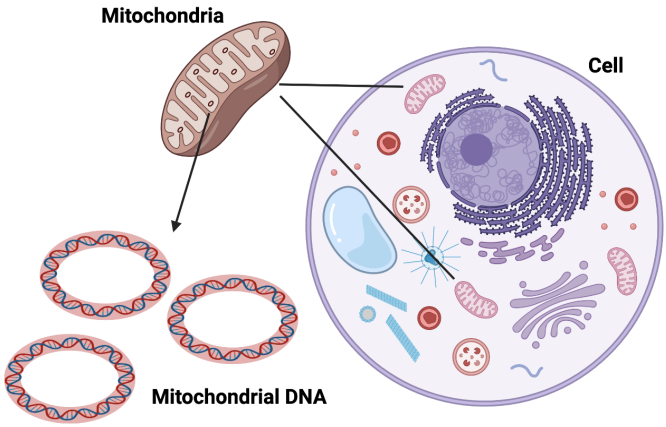


Figure 1: The mitochondrial genome is 16.6 kilobases long and is made of a circular DNA molecule. Multiple mitochondria are found in each cell.[5]

Mitochondria are responsible for the generation of more than 90% of the ATP in the typical cell, and they do this using the oxidative phosphorylation pathway.[6] This pathway is a critical metabolic process that takes place within the inner membrane of the mitochondria. The proteins that participate in making this pathway are called the electron transport chain and are primarily encoded in the mitochondrial DNA.[7] These protein chains create a gradient in the form of electrical and chemical potential energy across the inner mitochondrial membrane that is then harnessed by ATP synthase to transform ADP to ATP. The oxidative phosphorylation pathway is made up of 5 protein complexes, which are encoded by both the mitochondrial genome and the nuclear genome. The genes in the mitochondrial genome are thought to contribute to the necessary functions in the pathway, and genes that have moved to the nuclear genome are thought to encode many supportive and nonprotein complex encoding elements.[8] When there are

mutations that occur in these genes, the rate of ATP production can be impacted and is often decreased, which can have cascading deleterious effects.

Mutations that occur in somatic cells accumulate with age and have been associated with degenerative disease, specifically neurodegeneration and musculature degeneration in humans.[4,9] Inherited and accumulated mutations in mtDNA are impactful to energetic processes, and the gradual accumulation of somatic mtDNA mutations at different life stages impacts the function of all cellular processes.

1.2 Drosophila as a model organism

In humans, expansion of somatic mtDNA mutations directly impacts both aging and disease.[9,10] Investigating the mtDNA genome of Drosophila simulans allows for a more detailed examination of heteroplasmy.[11] Using Drosophila also assists with ease of data collection, as they are easy to breed, have fast generation times, and allow for whole organism sequencing.

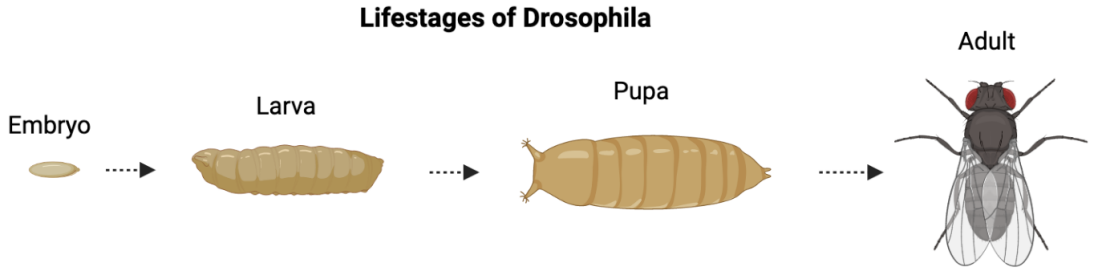


Figure 2: The life stages of drosophila are the embryo, larva, pupa, and adult. Mutations are somatic if they occur in the life stages after the embryo (the larva, pupa, or adult).[5]

By analyzing these mutations over life stages, a rate of somatic mtDNA mutation accumulation in drosophila can be estimated. The rate of mutations in the mitochondrial genome is reflective of the DNA damage that occurs and the repair mechanisms that are present. Understanding the rate and type of mutations that occur is a way to begin to quantify these mechanisms. Previous studies were limited by being unable to detect many low-frequency mutations, which could have significant impact on their results as a failure to see these mutations impedes accurate estimation of mutation distribution and frequency.[12] Selection against these mutations has yet to be shown in drosophila but has been seen in humans. Understanding the rate at which heteroplasmy accumulates and the underlying mechanisms by which it is created will provide insights into the diseases and disorders that are influenced by it.

In order to understand if mutations get selected against across life stages, we used data from ultra high accuracy sequencing to address our overarching hypothesis that deleterious mutations are purged as they accumulate over lifestage. This has yet to be shown in Drosophila, and by looking at the allele frequency, we have confirmed that we are looking at very low

frequency mutations that have not been seen previously. We use this data to view a mutation rate spectra and from that and an accumulation of mutations over lifestage, we can conclude that there is selective pressure against the accumulation of mutations over age.

2 Methods

2.1 Genome Sequencing

We performed ultra accurate sequencing on individuals collected from Drosophila simulans over three life stages, larva, pupa, and adults. Our sequencing method uses rolling circle amplification to create long repetitive concatemer molecules from ~100bp size-selected DNA fragments.[13] We extracted DNA from whole tissue samples and denatured it, then the resulting single-stranded DNA was sonicated into fragments that are approximately 100 bp in length. These fragments were then circularized and combined with a rolling circle amplification enzyme, Phi29 polymerase, and random primers. Our final product is a long double-stranded DNA sequence composed of the original approximately one hundred base pairs repeated many times over. These tandem copies are then sequenced with Illumina, and the resulting sequence contains the bases of the original circulated sequence and can then be used to construct a consensus sequence. These reads contain multiple copies of the repeated sequence, albeit rotated, from which we can reconstruct the original sequence. This allows near-complete elimination of errors introduced by Illumina sequencing and accurately identifies low-frequency mutations.[13]

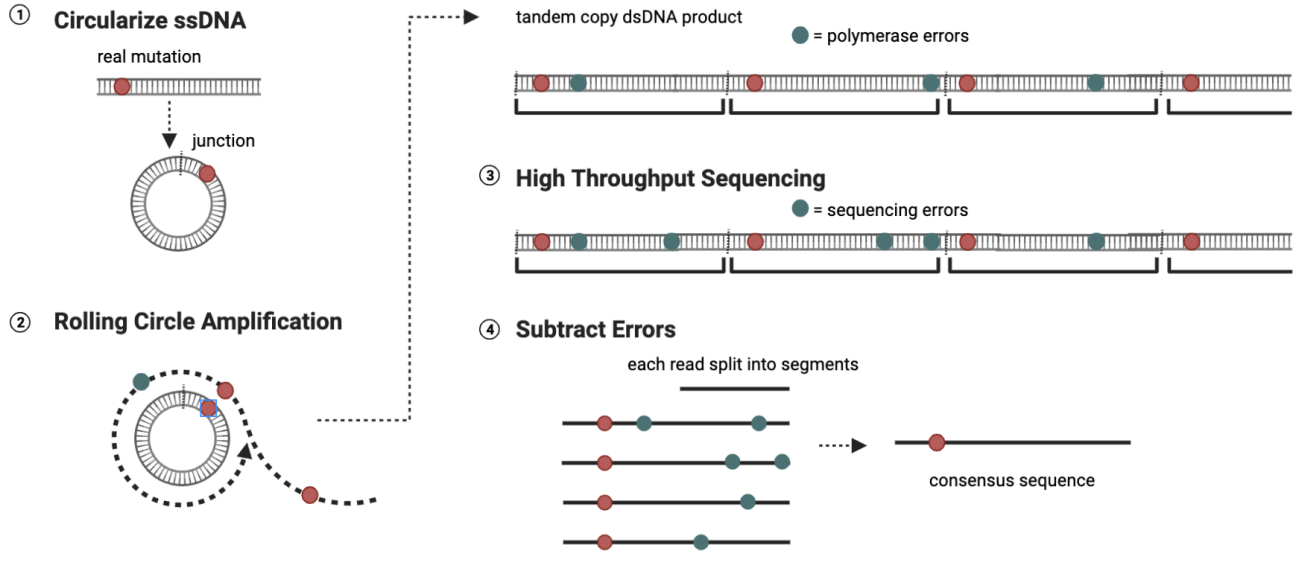


Figure 3: The pathway by which Circle Seq generates a consensus sequence from DNA. First, the DNA is denatured and single-stranded DNA is circularized, then random primers are annealed to circles, and Phi29 polymerase is used to perform rolling circle replication. These random primers and Phi29 polymerase turn long single-stranded copies into double-stranded DNA (Step 2). The copies are then run through a high-throughput sequencing machine (Step 3). The sequence is then aligned, and a consensus sequence is created (Step 4).[5,13]

2.3 Reconstruction

Our results from high throughput sequencing of the rolling circle amplified DNA were aligned to create consensus sequences. Then we performed global alignment with bwa mem, where the mapping location was extracted and split along the indices where the segments were trimmed by bwa.[14] We then realigned the resulting fragments to the reference surrounding the original mapping location with the Smith-Waterman local alignment algorithm as implemented in biopython.[15,16] Our final consensus sequence was compiled from these local alignments and stored with its mapping location as an entry in a bam alignment file. Then we constructed a pileup from consensus bams, which were flagged as QC pass/fail based on whether any single subsection fails to remap to the target region; reads which fail this are removed. The result of this genome reconstruction was a pileup file that contained the chromosome, position, ID, reference, alternate codon (the mutation), and the quality of the read. This can then be used to generate a data frame that can be used to view the mutations in the mitochondria.

2.4 Downsampling

Our ability to detect low-frequency variation is highly dependent on total genome coverage. Since most variation is rare and low-frequency and because there are millions of mitochondria in each individual, our ability to detect low-frequency variation is linear with total sequencing depth. To make counts across samples comparable, the lowest coverage samples were removed, then samples with higher mean depth were downsampled by randomly removing read coverage until all samples had the same mean depth. This allowed for an accurate comparison of counts of types of mutations and rate calculations.

2.5 Statistical testing

To analyze the impact of these mutations on the genes encoded in the genome, they were annotated for amino acid changes with the snpEff tool based on the latest public Drosophila simulans release.[17,18]

After annotation, a Ka/Ks ratio (ω) was computed. The Ka/Ks ratio is calculated using the ratio of the nonsynonymous substitution rate over the synonymous substitution rate.[19] Under neutral evolution without selection, the ratio is determined by simple mutation rates and $\omega = 1$. A ratio $\omega > 1$ suggests that there are more nonsynonymous substitutions than would be explained by mutation rates, resulting from positive or diversifying selection. Conversely, negative or purifying selection with $\omega < 1$ implies that mutations that alter the amino acid sequence are being

selected against.[20] We compute ω across whole genes to evaluate selection within somatic tissues.

3 Results and Discussion

3.1 Data Overview

We produced circle-seq libraries for nine reference strain Drosophila simulans, coming from three different life stages: larva, pupa, and adult. In total, we found 1657 somatic mutations across all individuals and with a median of 187 somatic mutations identified in each individual. The density of the number of reads varies slightly in each of the individuals sequenced, though the majority of the reads are across the whole genome and in each individual is very low frequency (Figure 5, Supplementary Figure 1).

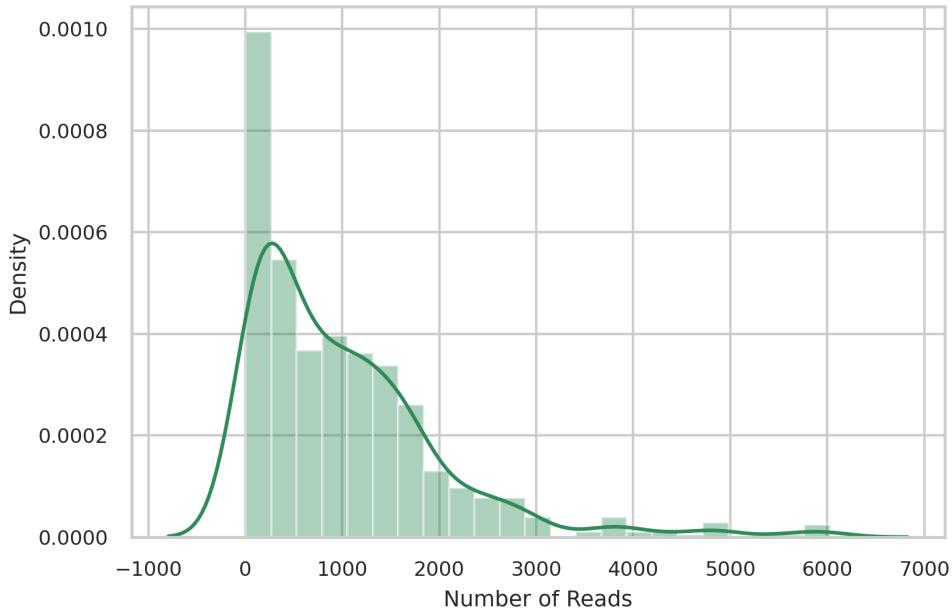


Figure 4: Mitochondrial mutation distribution of sequencing depth. This shows a density plot that indicates the distribution of the number of reads across every individual in the dataset. It shows the large number of low-frequency reads present in the data. Circle Sequencing allows for these low-frequency reads to be trusted as accurate and not due to sequencing error.

3.2 Allele Frequency

The frequency of mutations is skewed towards the very low end of the spectrum, and there are a large number of mutations that are at the lowest levels of detectable mutations. The binned log circle frequency spectrum is the number of consensus reads which contains an alternative divided by total coverage at that site for each mutation. The majority of the frequencies are under 10, meaning that the consensus reads that contain an alternative allele divided by total coverage at that site for each mutation are low (Figure 5).

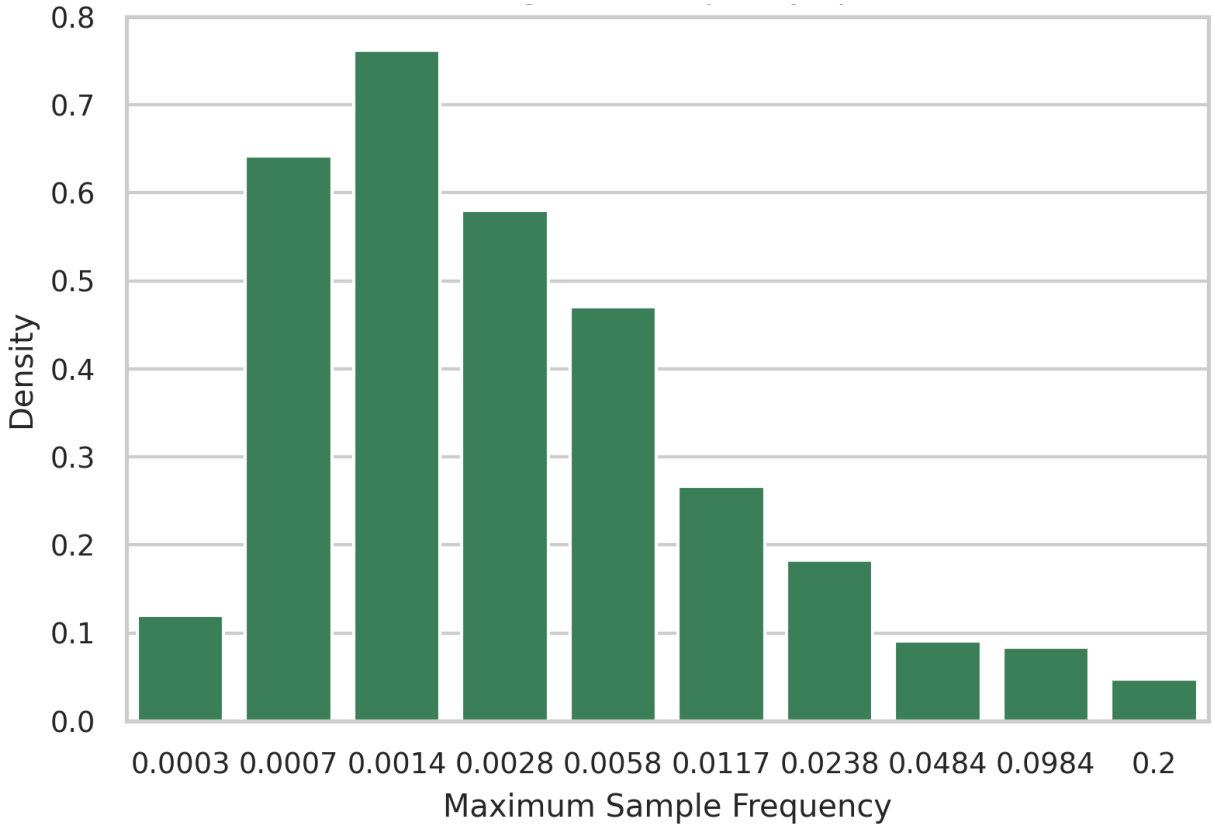


Figure 5: Mitochondrial mutation allele frequency spectrum. Histogram of log-transformed circle frequencies. This is the number of consensus reads which contain an alternative divided by total coverage at that site for each mutation. The majority of the mutations skew towards the lower frequency side of the spectrum.

3.3 Mutation Rate Spectra

The mutation rate spectra is derived from the nucleotide composition of mtDNA and inherent biases in DNA damage and repair. The mitochondria of insects have high AT content in their genomes and have substantial strand bias.[3,21] The major strand is the coding strand for nine of the proteins in the oxidative phosphorylation pathway.[22]

The two strands display slight differences in the mutation spectrum though there is an enrichment of transition mutations across expected paired mutation types (Figure 6).[12] This data represents both the major and minor coding strands, which accounts for some of the lack of symmetry across paired mutation sites. 72.5% of the somatic mutations found are mutations that involve A and T, which also explains the lack of symmetry.[23] This value is in agreement with the literature.[3] Mutational biases of T → C and C → T are indicative of sequencing on the major strand; the spectra confirms that the data likely contains content from this strand.[12,24] Importantly,

it is not clear why a source of sequencing error would display strand-specific biological asymmetry.

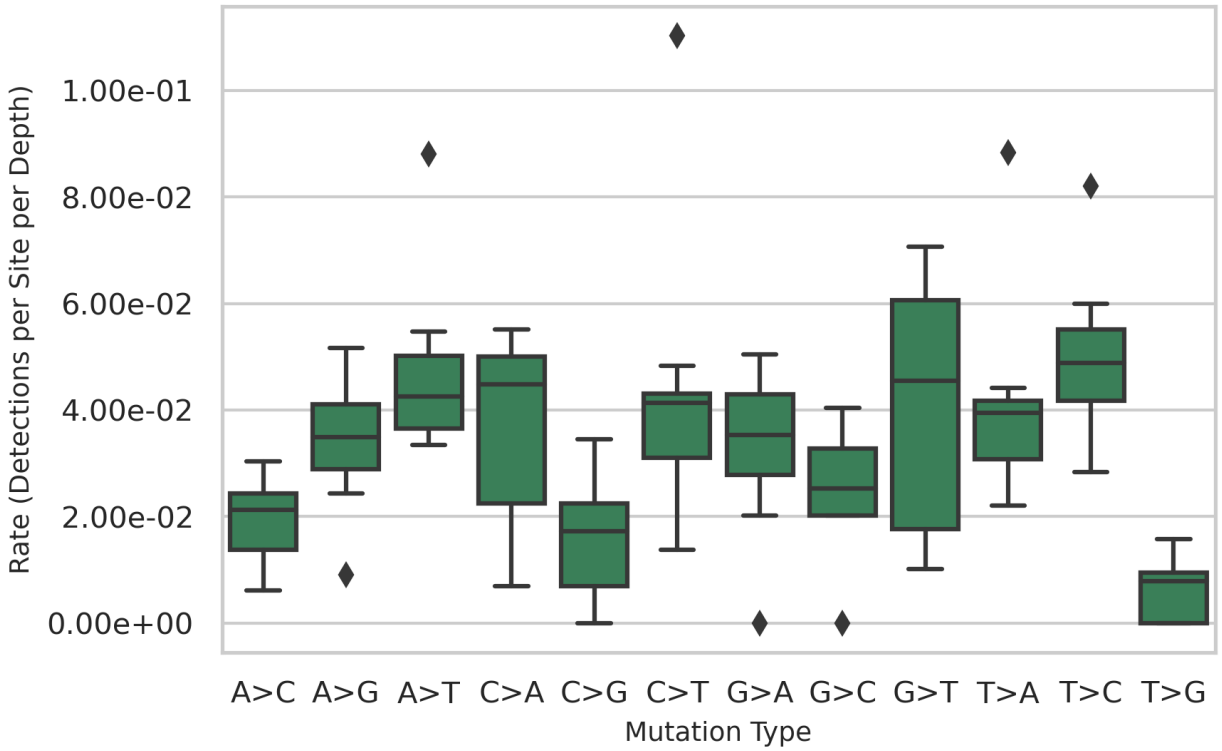


Figure 6: Somatic mutation rates in the mitochondrial genome across all life stages. The rate of detections per site per read covering that site, for each individual, of different mutation types across the entire genome. There is relative symmetry across mutation types for the mtDNA genome.

The likelihood of oxidative DNA damage occurring in the sequencing process is unlikely due to the similarities in the rates of G→T mutations and C→A mutations occurring, which when reciprocal do indicate a likely error in sequencing.[25] The use of double-stranded DNA processing technologies helps to yield a more balanced spectrum of these mutation frequencies (Figure 6).

3.4 Mutation Accumulation over Life stages

We found a rapid increase in the presence of mutations over the life stages in the mitochondria, which is indicative that the mitochondrial genome is mutating with drosophila age (Figure 7). The similarities in larval and pupal mutation counts are likely due to the larva being collected at the 3rd instar stage, which is very close in time to the pupal stage. This increase with lifestage supports the hypothesis that the increase in mutations is likely a driving force in mitochondrial disease and dysfunction, as those diseases tend to present primarily in organisms towards the end of their lives.[26,27]

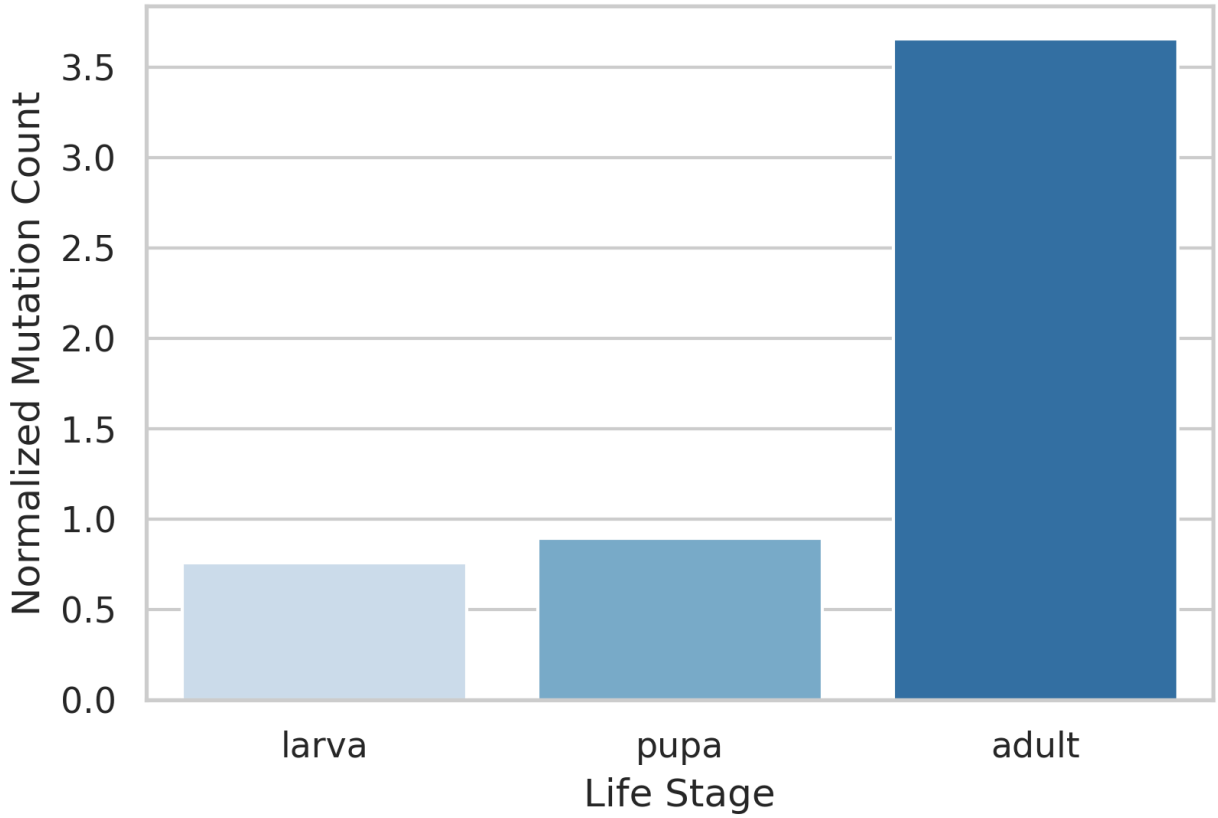


Figure 7: The count of mutations over each lifestage normalized for the total count of individuals after downsampling for depth. Adults show notably increased heteroplasmy compared to earlier life stages.

3.5 Selection Against Missense Mutations

The life stage-averaged genome-wide Ka/Ks score was 0.3475, which indicates that there is negative selection acting upon mtDNA within somatic tissues as an organism ages. Looking at the protein-coding genes in the mitochondrial genome, the genome-wide Ka/Ks value for each lifestage was 0.4257 for the larva, 0.3764 for the pupa, and 0.2373 for the adult. This suggests that there may be a selection event that occurs during the transition from larva to adult, and there is selection that is occurring, as there are fewer nonsynonymous mutations than synonymous mutations from in the larva than the pupa ($p < 0.00064$, Fisher's exact test), and a similar pattern from the pupa to the adult ($p = 0.028$, Fisher's exact test).

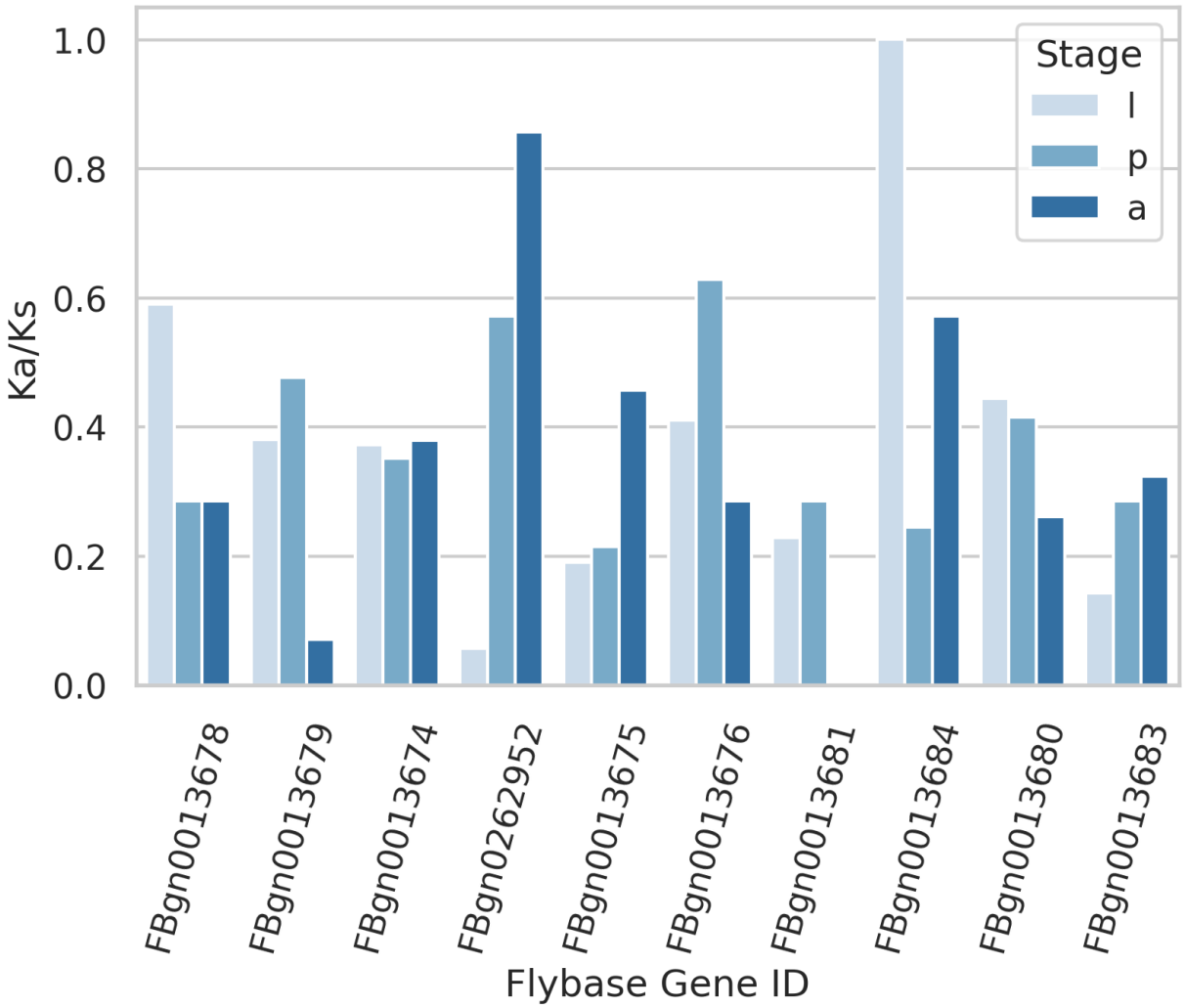


Figure 8: The Ka/Ks scores for each gene across each lifestage. The stages shown are larva, pupa and adult.

The genes in which the Ka/Ks scores decrease over lifestage, there is a sign that there is a change in the pressure of selection against the accumulation of nonsynonymous mutations over the life stages.[28] This is most clear in the mitochondrial NADH-ubiquinone oxidoreductase chain 2 gene (FBgn0013680), in which there are progressively lower Ka/Ks scores in each lifestage. This gene encodes for the core subunit of the mitochondrial membrane respiratory chain NADH dehydrogenase, which is responsible for the transfer of electrons from NADH to the respiratory chain.[29] The mitochondrial-DNA-encoded NADH dehydrogenase subunit 2 is part of the first complex of the mitochondrial respiratory chain referred to as Complex 1. Previous studies have shown that mutations in this mitochondrially encoded gene are the hallmark of many mitochondrial diseases.[9] This subunit participates directly in the proton pumping mechanism of complex I, and mutations occurring in this subunit are lined to significantly diminished respiratory champion activity.[30]

The gene that appears to have the least selection pressure against the accumulation of nonsynonymous mutations, is a gene that encodes the mitochondrial-DNA-encoded NADH dehydrogenase subunit 4 (FBgn0262952). This gene is thought to encode one of 11 helicases that are involved with coupling mechanisms. The accumulation of mutations in this gene has not been linked to mitochondrial disorders, though its function is necessary for high activity in the oxidative phosphorylation pathway.[31] This pathway does not appear to have any genes in which non synonymous mutations are not selected against to some degree; this is in alignment with the literature, which suggests that if a gene is found in the mitochondria it is because it has been deemed essential to the function and processes of the oxidative phosphorylation and genes that are nonessential undergo nuclear drift.

4 Conclusion

Since the discovery of mtDNA 25 years ago, mitochondrial mutations have been identified as a cause of human diseases. These diseases frequently become more common with age, especially if there is an increase of heteroplasmy of the mitochondria. Polymorphisms in mtDNA have been linked to multiple neurodegenerative disorders, cancer, and muscle degeneration. These mutations seem to preferentially collect in organs that are of high energy use, which is often correlated with cells that perform essential functions. The type of evolutionary pressure these genes are subjected to was previously unknown. Mitochondrial heteroplasmy has been identified as harmful, but there has been little research into investigating the selection events and pressure that may impact the types and rate of mutations occurring.

I have found that there is an increase in mitochondrial DNA mutations across the life stages of Drosophila. This increase aligns with the literature hypothesis and confirms that mutations can be attributed as a cause of mitochondrial disease, as they collect with age. The ultra accurate sequencing method is used to mitigate as many potential mutations as remnants of sequencing error. The mutation rate spectra confirms that the mutations shown in this data align with the literature and can be used to confidently direct and analyze the types of mutations occurring and the effects these changes have on specific genes.

My analysis revealed that there was selective pressure in the mitochondria against non synonymous mutations. The genes that are shown to be undergoing the most selective pressure against these mutations appear to be major contributors to the function of the mitochondrial respiratory chain, which provides strong evidence that there is non-random selection. This negative selection demonstrates that the cell is shaping the extent of heteroplasmy in its mitochondria. I postulate that there is not likely to be a distinct bottleneck in the life span of drosophila, but there may be pressure across the lifespan that impacts the accumulation of mutations.

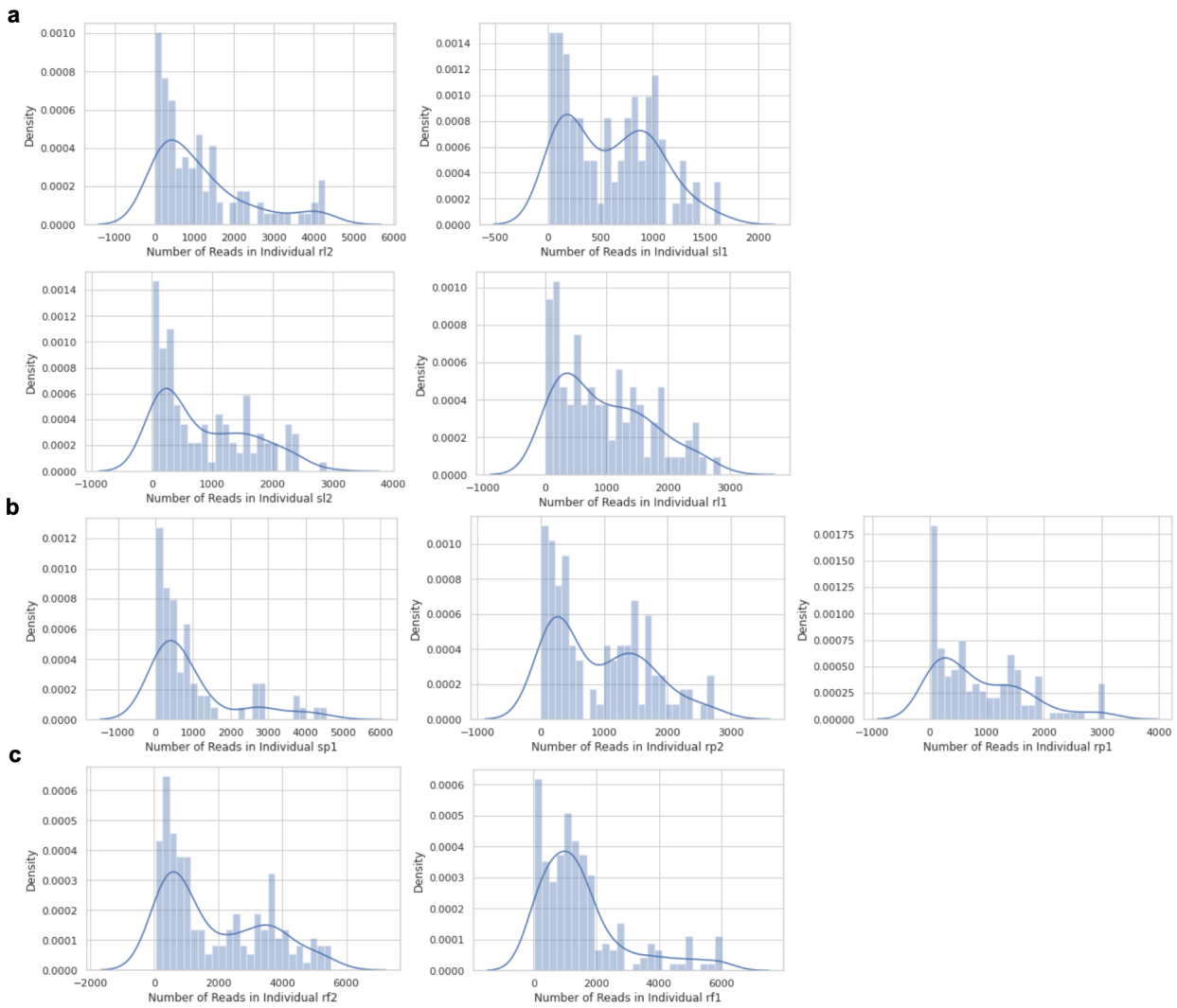
5 Future Work

In future work I intend on investigating more into the implications of which genes are shown to be undergoing the most selective pressure. The functions of these genes as they contribute to the creation of ATP has been investigated, but a deeper understanding of which polymorphisms are tolerated by the cell versus which are selected against would be important for determining the impacts on disease. I plan on determining a mutational signature analysis of the mutations across each of the life stages to gain a better understanding of the mechanisms by which heteroplasmy accumulates. Using this workflow to evaluate levels of heteroplasmy would allow future experiments to be performed in which I could use treatments, such as early life stage heat shock or starvation, and determine their effects on the life-long accumulation of mitochondrial mutations.

This method can also be used to test for the mechanisms and specific timing that selection occurs in the mitochondria. I intend on using it to create a larger data set with each lifestage being specifically aged in multiple stages, which can then be used to get an even more detailed view of the ways in which non synonymous mutations are selected against. This could elucidate the process that the mitochondria undergo after the genetic bottleneck at oogenesis, including any potential bottlenecks or notable changes in selection associated with metamorphosis. These discoveries could provide even more insight into the development and progression of mitochondrial disorders in drosophila and other organisms.

6 Supplementary Figures

Over each individual, there is a mutation distribution of depth of the number of reads. The density of the reads does vary for each individual sequence, but the large proportion of the number of reads are very low frequency (Supplementary Figure 1). Individuals vary in their maximum number of reads. The largest spike in every individual is before 1000 reads. Circle Sequencing allows for these low-frequency reads to be trusted as accurate and not due to sequencing error. The distribution of the depth for all the samples supports the conclusion that the vast majority of reads are found to be low frequency (Figure 4).



Supplementary Figure 1: The mutation distribution of depth of the number of reads over each individual, grouped by lifestage. These show a density plot that indicates the distribution of the number of reads across every individual in the dataset. It shows the large number of low-frequency reads present in the data. Section A shows pupa, Section B shows larva. The naming of these samples is that the first letter is decided from the batch of flies, the second letter is the life stage and the third is a count of the number of individuals that are from that bath at that life stage.

7 GitHub

The code used in this project, to downsample, anayze and graphically represent the data can be found at https://github.com/maracbaylis/Mitochondria-Project.

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