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SYMPOSIUM

An Ecologist's Guide to Mitochondrial DNA Mutations and Senescence

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Synopsis Longevity plays a key role in the fitness of organisms, so understanding the processes that underlie variance in senescence has long been a focus of ecologists and evolutionary biologists. For decades, the performance and ultimate decline of mitochondria have been implicated in the demise of somatic tissue, but exactly why mitochondrial function declines as individual's age has remained elusive. A possible source of decline that has been of intense debate is mutations to the mitochondrial DNA. There are two primary sources of such mutations: oxidative damage, which is widely discussed by ecologists interested in aging, and mitochondrial replication error, which is less familiar to most ecologists. The goal of this review is to introduce ecologists and evolutionary biologists to the concept of mitochondrial replication error and to review the current status of research on the relative importance of replication error in senescence. We conclude by detailing some of the gaps in our knowledge that currently make it difficult to deduce the relative importance of replication error in wild populations and encourage organismal biologists to consider this variable both when interpreting their results and as viable measure to include in their studies.

Introduction

With the rise of complex animal life, a novel division among cell lines evolved (Extavour 2007; Radzvilavicius et al. 2016). The germline was designated to never differentiate and to be the only cells to engage in sexual reproduction. In contrast, cells in somatic lines underwent increasing differentiation and specialization as more complex lifeforms evolved, but they were barred from sexual reproduction (Extavour 2007). Hence, the role of the germline is to produce gametes and maintain genetic lineages—both mitochondrial and nuclear—in perpetuity. The function of the soma, in all of its complexity and diversity, is to propagate germ cells (Extavour 2007; Vijg 2014; Hill 2019). The perpetuation of each germline is contingent upon the success of its soma, and the success of a soma arises within the context of changing and unpredictable environments and with endogenous and exogenous constraints. One key endogenous constraint is senescence, the gradual deterioration of a cell's functional characteristics with age (Ricklefs 2008). The somas of essentially all bilaterian animals decline with age, and this decline potentially imposes critical constraints on lifetime reproduction (Monaghan et al. 2008). The pace of decline varies dramatically across species; as a consequence, the fitness of most animals is highly dependent on the timing of the onset and the rate of senescence (Kirkwood and Rose 1991; Nussey et al. 2008).

Because survival and fecundity are the currencies of fitness, senescence is a central topic of investigation for many evolutionary ecologists (Kowald and Kirkwood 2015; Lemaître et al. 2015). Across the tree of life, organisms display enormous variation in the lifespan of their somas (Jones et al. 2014), and investigations of the factors that contribute to these differences in longevity can be addressed from two different perspectives. Investigations that adopt an ecological perspective emphasize the exogenous forces that drive selection (Monaghan et al. 2008; Gaillard and Lemaître 2017). Alternatively,

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investigations that adopt a mechanistic perspective focus on identifying changes at a molecular and cellular level that ultimately determine the longevity of both individuals and species (Hughes and Reynolds 2005; Childs et al. 2015). Ecologists tend to study senescence from the perspective of life-history evolution and only secondarily invoke the mechanisms that might underlie aging. The mechanism for aging most frequently stated by organismal biologists is mitochondrial-derived free radical damage (i.e., oxidative damage; Harman 1956; Liochev 2013), and it is becoming increasingly common for whole-animal studies of non-model species to include measures of mitochondrial respiratory performance (Bize et al. 2014; Jimenez et al. 2014; Salin et al. 2016; Mowry et al. 2017; Zhang et al. 2018).

While evolutionary ecology has embraced the idea that damage from free radicals from mitochondria is the primary cause of mitochondrial DNA (mtDNA) mutations and a key contributor aging (Selman et al. 2012; Speakman et al. 2015), biomedical research has been engaged in an intense debate about the sources of mtDNA mutations, as a mechanism that underlies aging. Specifically, observations from several empirical studies have led some authors to conclude that errors arising during the replication of the mitochondrial genome play a substantially greater role in mtDNA mutation rate and senescence than damage from free radicals (Larsson 2010; Melvin and Ballard 2017; Szczepanowska and Trifunovic 2017). Other authorities question whether mtDNA mutations originating from any source are sufficient to have a significant impact on bioenergetic capacity and the rate of senescence (Vermulst et al. 2007; Kauppila et al. 2018; Ma et al. 2018). On the contrary, many researchers continue to see a role for oxidative stress in aging processes (Gil del Valle 2011; Cui et al. 2012).

The goal of this review is to introduce evolutionary and physiological ecologists to replication error as a potential source of mtDNA mutations, to review evidence that replication error plays an important role in senescence, and to consider evidence suggesting mtDNA mutations may not contribute significantly to senescence. We also highlight key gaps in understanding mtDNA mutation rate which continue to make it difficult to deduce the relative importance of replication error to performance in wild populations.

Mitochondrial performance and sources of mtDNA mutations

Mitochondria produce ~95% of the adenosine triphosphate (ATP) required to support the energy demands of animals (Tzameli 2012). The mitochondrial performance of a tissue arises from the capacity for oxidative phosphorylation (OXPHOS) by each mitochondrion multiplied by the number of mitochondria in that tissue (Brand and Nicholls 2011). Several variables can impact OXPHOS including, but not limited to, the function of subunits of the electron transport system complexes (Bar-Yaacov et al. 2012; Hill 2015), the relative number of OXPHOS complexes (Swalwell et al. 2011), the capacity of the mitochondria to optimize their ATP production (Celotto et al. 2011; Westermann 2012; Genova and Lenaz 2015), and the integrity of mitochondrial lipid membranes (Bindoli 1988). As a consequence, any loss of function by mitochondria can directly impact individual performance (Coen et al. 2012), and numerous studies have found significant negative correlations between the age of an animal and the performance of its mitochondria. In humans, for example, mtDNA abundance and mitochondrial ATP production in skeletal muscle, measures of respiratory performance (the respiratory control and P/O ratios) in the liver mitochondria, and the abundance of Complex IV in the brain all decline linearly with age in concert with decline of muscle performance, liver function, and cognition (Yen et al. 1989; Ojaimi et al. 1999; Short et al. 2005). Because mutations to mtDNA can directly alter the capacity of the cell to both produce ATP and make additional mitochondria, several investigators hypothesize the accumulation of mutations to mtDNA is a critical contributor to aging phenotypes (Kauppila et al. 2017; Melvin and Ballard 2017).

There are two potential sources of mtDNA mutations: damage from free radicals and replication error (Lagouge and Larsson 2013). Mutations caused by free radicals are most familiar to ecologists. Briefly, free radicals are compounds with an unpaired valence electron. The majority of free radicals in cells that interact with DNA are produced endogenously (Halliwell and Gutteridge 2015). During OXPHOS, a small percentage of electrons (~0.1%) are lost from the electron transport system, and these "leaked" electrons readily react with oxygen to make superoxide, a reactive oxygen species (ROS) (Hansford et al. 1997; Nicholls and Ferguson 2013). Most of the electron loss occurs either at Complex I, leading to the formation of superoxide in the mitochondrial matrix, or at Complex III, leading to the formation of superoxide in both the matrix and the mitochondrial intermembrane space (Murphy 2009; Brand 2016). Superoxide, in turn, can react with nitrous oxide to produce peroxynitrite, a reactive nitrogen species (RNS)

(Murphy 2009). While ROS are typically far more abundant than RNS, both can directly or indirectly damage lipids, proteins, and DNA if intracellular antioxidants do not intervene (Halliwell and Gutteridge 2015; Zhang and Hood 2016). Accumulation of cellular damage occurs when oxidative damage levels exceed the capacity for repair or when repair processes begin to fail.

Damage from ROS can include peroxidation of lipids in membranes (Pacifici and Davies 1991) and in extreme cases, advanced lipid peroxidation end products can contribute to cellular dysfunction (Pamplona 2008). Protein oxidation can alter protein function, contribute to protein fragmentation and aggregation, and cause endoplasmic reticulum stress which can further elevate ROS (Berlett and Stadtman 1997; Malhotra and Kaufman 2007). ROS can also interact with and change DNA and contribute to the accumulation of mutations (Jena 2012; Halliwell and Gutteridge 2015). ROS and damage resulting from free radicals are abundant in senescent cells (Passos et al. 2010).

All forms of ROS damage—to lipids, proteins, and DNA—have the potential to be repaired. Past studies have estimated that endogenous ROS generate 50,000 DNA mutations per human cell per day (Lindahl 1993; Maynard et al. 2015). Oxidative damage to mtDNA, lipids, and proteins accumulate when repair mechanisms cannot keep up with an increase in the rate of mutations or when there is a reduction in the production of effective repair enzymes such as 8-oxoguanine DNA glycosylase (OGG1) (Maynard et al. 2015). Damage that is not repaired can have persistent negative impacts on the performance of cells and tissues (Monaghan et al. 2009; Speakman and Garratt 2014; Blount et al. 2016; Zhang and Hood 2016). Levels of ROS in tissues and the accumulation of damage from ROS have been shown to correlate with lifespan both at the intra- (Forster et al. 1996; Bize et al. 2008; Gan et al. 2012) and interspecific level (Sohal et al. 1990; Ku and Sohal 1993; Ku et al. 1993; Herrero and Barja 1998; Barja and Herrero 2000; Speakman et al. 2004; Lambert et al. 2007; Barja 2013). Despite these observations, recent works suggest the relationship between ROS and mitochondrial performance is complicated because ROS are not consistently harmful. Mitochondria in cells often display a biphasic response to exposure to ROS, a pattern that is called mitohormesis. Under mitohormesis, ROS improve mitochondrial performance by increasing mitochondrial density and upregulating antioxidants and repair when they are produced at modest levels. When ROS is produced at high levels, oxidative damage can accumulate as describe previously (Zhang and Hood 2016; Hood et al. 2018; Zhang et al. 2018).

While many ecologists focused their effort on oxidative damage as a potential source for mtDNA mutations, cell and molecular biologists increasingly focused on replication error as a major source for mtDNA mutations (Zheng et al. 2006; Larsson 2010; Kennedy et al. 2013; Itsara et al. 2014; DeBalsi et al. 2017). Replication of the mtDNA molecule is necessary for the regular renewal of the mitochondrial population, a process called mitochondrial biogenesis. The production of new mitochondria allows for the adjustment in the energy capacity of tissues as well as for compensation for underperforming mitochondria. During mitochondrial biogenesis, duplicate strands of the double-stranded mtDNA are synthesized, errors are corrected, and the new plasmid is then encapsulated as the new organelle is formed by fission (Clayton 2000; Fig. 1). The polymerase primarily responsible for replicating the mtDNA plasmid is mitochondrial polymerase subunit gamma (POLG). POLG is nuclear-encoded and, once transported into the mitochondrion, it functions both in replicating the old and correcting errors in the new mtDNA molecule. During the mtDNA replication process, a mitochondrial helicase, twinkle (TWNK), is recruited to the origin of replication. The original heavy (H-strand) and light strands (L-strand) of mtDNA are separated by TWNK as POLG works to synthesize new nucleotides to develop the new H-strand. Mitochondrial single-stranded binding proteins (mtSSBs) bind to the displaced parental H-strand to prevent it from recombining during replication. When the replication machinery (replisome) reaches the origin of replication for the L-strand (O_L) mtSSBs lose their affinity to bind to the O_L region allowing for the DNA-directed RNA polymerase (POLMRT) to bind and begin primer synthesis on the L-strand and for POLG to begin synthesis of the L-strand in the opposite direction. A topoisomerase works to relieve supercoils and tension due to replication ahead of the replication fork. A catenated plasmid product forms and another topoisomerase decatenates it, leaving new and old identical copies of mtDNA plasmid. These copies can then be divided among two mitochondria during fission (Falkenberg et al. 2007; Gustafsson et al. 2016; see for detailed reviews).

Most eukaryotes are thought to have a single mitochondrial genotype at fertilization (Lagouge and Larsson 2013), a condition referred to as homoplasmy. Even if homoplasmy is achieved at conception, however, this homogeneity is short-lived. As mitochondria are replicated to populate the cell lines

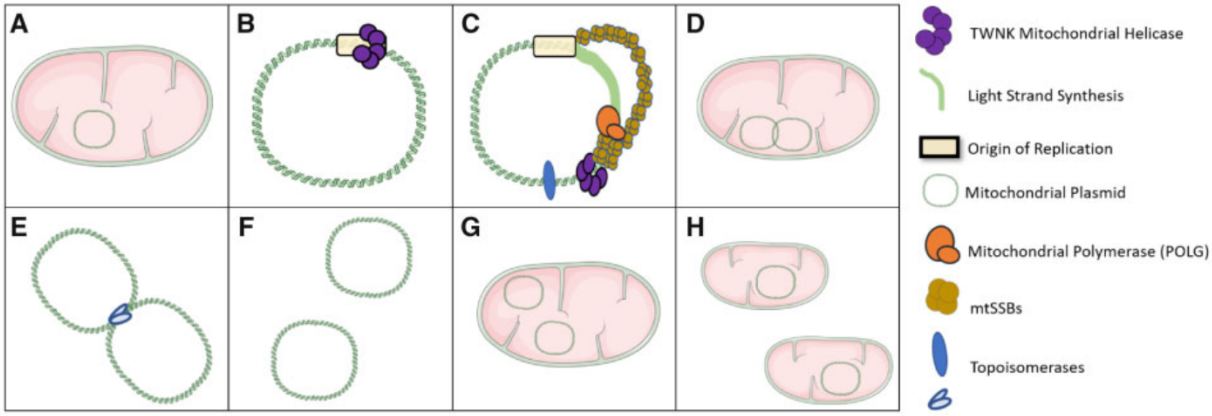

Fig. 1 The process of mtDNA replication. (**A**, **B**) the recruitment of mitochondrial helicase, TWNK, to the origin of replication during initiation. (**C**) A moving replisome as TWNK separates the H-strand and L-strand of mtDNA and POLG works to synthesize new nucleotides to develop the new H-strand, and mtSSBs bind to the displaced parental H-strand to prevent it from recombining during replication. When the replisome reaches the origin of replication for the L-strand (O_L) mtSSBs lose their affinity to bind to the O_L region allowing for POLMRT to bind and begin primer synthesis on the L strand and for POLG to begin synthesis of the L-strand in the opposite direction. A topoisomerase works to relieve supercoils and tension due to replication ahead of the replication fork. (**D**) Catenated plasmid product from replication. (**E**) Topoisomerase decatenates the plasmids. (**F**, **G**) Two distinct plasmids within the mitochondrial matrix. (**H**) Two mitochondria containing identical copies of mtDNA plasmids following fission.

of a bilaterian individual, mutations inevitably cause changes to the nucleotide sequence creating divergent mitochondrial lineages. Thus, heteroplasmy inevitably arises in the somatic cell lines of all individual bilaterians and it increases with age (Payne et al. 2013). Generally, single point mutations (SNPs) are most common, but deletions arising from single-stranded breaks, double-stranded breaks, and slips during replication are frequent forms of mtDNA mutation (Macey et al. 1997; Taylor and Turnbull 2005; Lagouge and Larsson 2013). Replication error induced SNP's are thought to primarily be associated with incorrect nucleotide pairing and slip deletions (Fig. 2).

There are several areas of interaction between the products of nuclear and mitochondrial genes (a.k.a. mitonuclear interactions), including Complexes I, III, IV, and V of the electron transport system, the mtDNA plasmid and its nuclear-derived transcription machinery and its nuclear-derived replisome, and the nuclear-derived aminoacyl transfer RNA (tRNA) synthase and the mitochondrial-derived tRNA which interact during translation (Fig. 3). Mutations in any of these mitochondrial and nuclear products at their point of interaction can hinder their performance. Errors which arise during replication have the potential to alter the efficiency by which these mitochondrial-derived and nuclear-derived products interact (Hill 2019). For example, mutations in the promoter regions of mtDNA can prevent or reduce the capacity of POLG to bind to the initiator regions of mtDNA (Ellison and Burton 2008, 2010). In addition, a mutation in the region coding for ribosomal RNA or tRNA can reduce or alter their capacity to produce mitochondrial proteins (Hoekstra et al. 2013; Adrion et al. 2016), and a mutation in key coding regions of OXPHOS proteins can hinder their compatibility with nuclear proteins and their capacity to support efficient ATP production (Sackton et al. 2003; Barreto et al. 2018). Repair processes that maintain the integrity of the mitochondrial genome are less efficient than the repair processes that maintain the nuclear genome (Larsson 2010; DeBalsi et al. 2017). Furthermore, mitochondria turn over at a higher rate than somatic cells, necessitating greater replication of mtDNA than nuclear DNA (nDNA). These factors lead to the gradual accumulation of replication errors within the DNA of a mitochondrial population over time.

Despite the potential impact mutations can have on the performance of an individual mitochondrion, many of the mutations which occur are synonymous SNPs or occur in areas of little functional consequence. Cells do an excellent job of guarding against loss of cell function. Each cell in an individual commonly carries 100s to 1000s of mitochondria and each mitochondrion can carry multiple genomes (Larsson 2010). Because mtDNA plasmids are so numerous, functional mitochondria are generally able to compensate for dysfunctional mitochondria (Larsson 2010; Lagouge and Larsson 2013; Kauppila et al. 2017). Animals typically maintain high levels of performance with modest levels of heteroplasmy (Payne et al. 2013; Kauppila et al. 2017). When a high proportion of mitochondria carry a mutation that alters

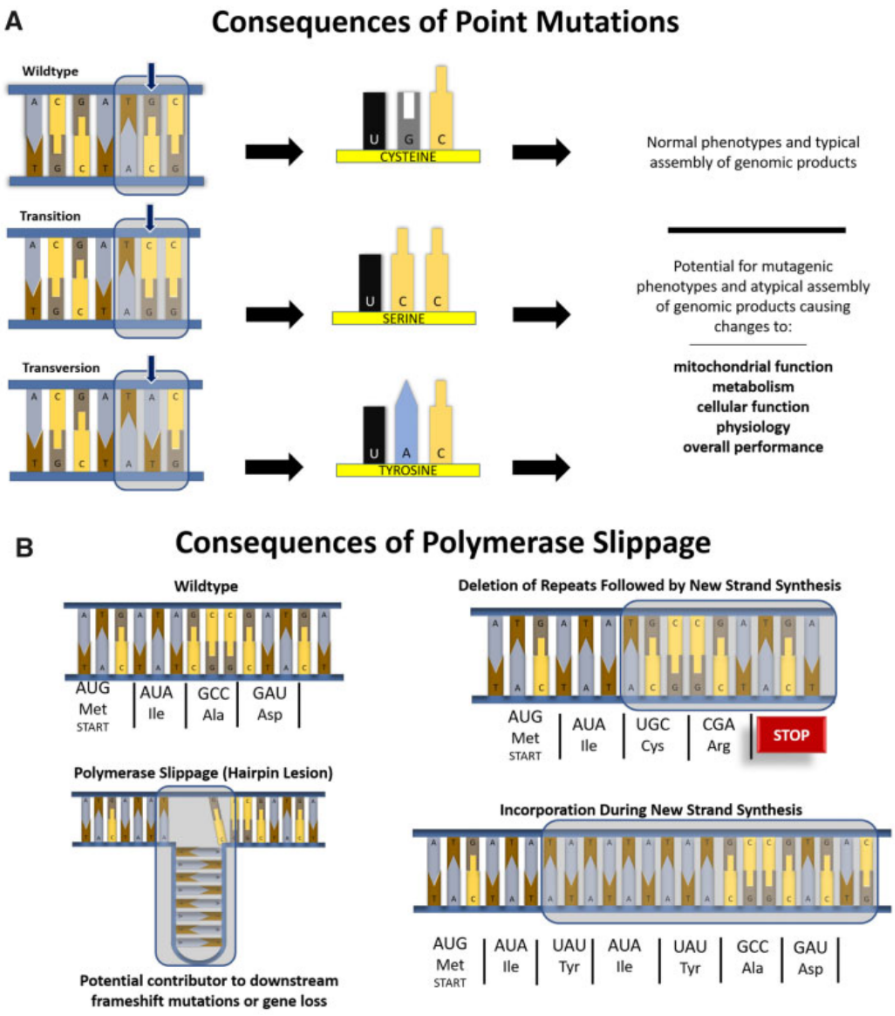

Fig. 2 Consequences of mitochondrial point mutations and slippage. (**A**) An example of the change in gene products and possible change in function due to transition and transversion point mutations versus wildtype. (**B**) Potential consequences of polymerase slippage. Among the examples presented are nonsense mutations from deletions and frameshift mutations from insertions, all of which ultimately influences protein expression.

function, however, compensation is no longer effective (Larsson 2010; Lagouge and Larsson 2013). For example, human patients with chronic progressive external ophthalmoplegia (CPEO) display large scale deletions in their mtDNA associated with a mutation in the mtDNA polymerase or associated replication machinery (Deschauer et al. 2007). To study the impact of these deletions on the energetic capacity of affected tissues, Hayashi et al. (1991) crossed the cells recovered from CPEO patients with cultured HeLa cells. The mitochondria in these cybrid cells did not display a decline in bioenergetic capacity until 60% of the mitochondria in the tissues carried deletions, as indicated by a reduction in cytochrome c oxidase activity. More abundant mutations are likely required for smaller deletions and SNPs. For example, Chomyn et al. (1992) found that >90% of mitochondria must carry a consistent SNP in the binding site for mitochondrial transcription termination factor before a reduction in oxygen consumption is detected in tissues cultured from mitochondrial encephalomyopathy, lactic acidosis, and stroke-like episode (MELAS) patients with the maternally inherited encephalopmyopathy syndrome is the $tRNA^{Leu}$ gene mutant. When mutations are random, as occurs with replication error or ROS-induced oxidative damage, it is likely that the bioenergetic capacity of a tissue would not be comprised until a much higher mutational load is achieved.

Does ROS or replication error play a more important role in mtDNA mutations?

Replication error and oxidative damage change mtDNA in different ways, which provides clues to

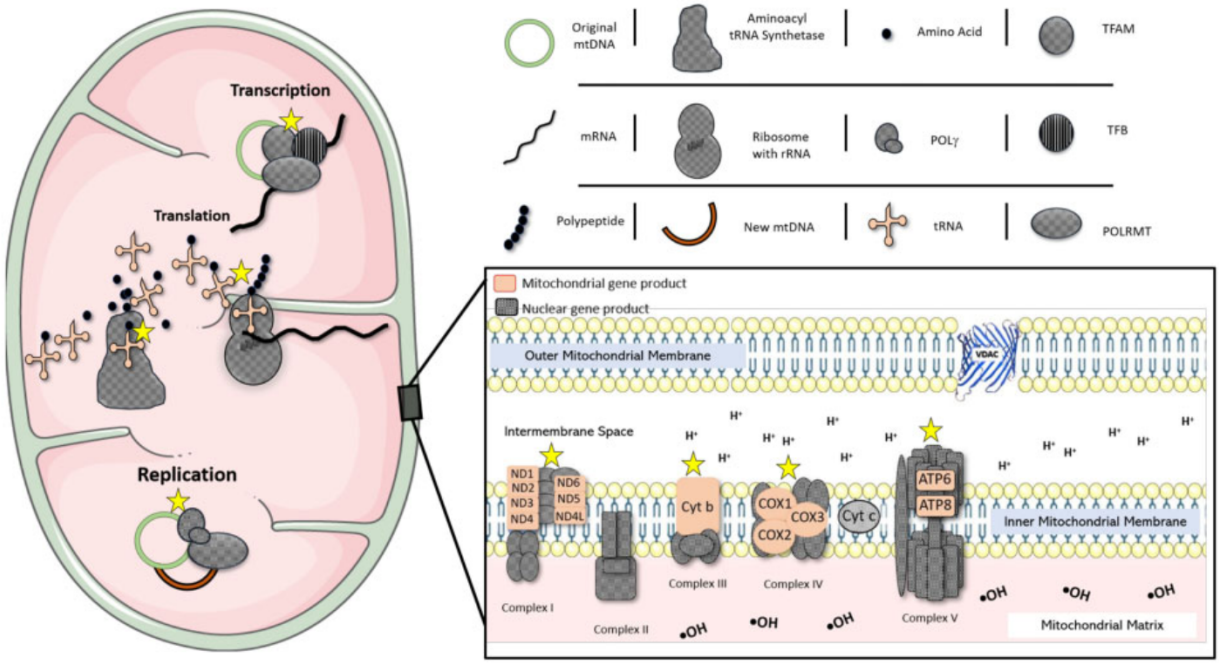

Fig. 3 Mitochondrial and nuclear products functioning within the mitochondrion. All non-textured proteins are coded by the mitochondrial genome and all textured proteins are coded by the nuclear genome. The stars within the figure represent locations of mito–nuclear protein–protein, protein–RNA, and protein–DNA interactions that exist, and which could be hindered when replication error mutations occur. TFA and TFB, transcription factors A and B; VDAC, voltage-dependent anion channel. Also depicted is VDAC, which functions in the transport of ions and metabolites like ATP and in regulating apoptosis.

researchers about which process may have been most important in creating a pattern of change in mtDNA. When ROS react with either mtDNA or nDNA, the most common reaction observed is with the nucleobase guanine. ROS-mediated oxidation of guanine produces 8-oxoguanine, a molecule that differs from guanine by only a few atoms (Banerjee et al. 2005). The repair of 8-oxoguanine by OGG1 releases 8-hydroxy-2'-deoxyguanosine (8-oxo-dG) as a byproduct. If left unrepaired, the damaged pyrimidine (guanine) gains higher binding affinity for adenine instead of cytosine resulting in a single point G:C to T:A transversion mutation (Brieba et al. 2004; Fig. 4). Replication error also has a common signature. Again, during replication guanine is more prone to mutation than other nucleotides and is commonly recognized as the alternative single-ring pyrimidine, adenine. If such a misrecognition occurs, then it is subsequently paired with another two-ring purine thymine instead of cytosine resulting in a G:C to A:T transition (Spelbrink et al. 2000; Fig. 4).

The development of the random mutation capture assay, or random mutation capture (RMC), allowed investigators to both estimate the percent of mtDNA nucleotides expressed as a mutant and estimate the proportion of nucleotide transitions and transversions (Bielas and Loeb 2005; Vermulst et al. 2008; Itsara et al. 2014). When applying the RMC assay to a given tissue, a subpopulation of mitochondria from each sample is evaluated. Polymerase chain reaction (PCR) is performed to amplify select segments of the mtDNA on individual mitochondria within the population of mitochondria isolated from each tissue. For each sample, the proportion of the unamplified segments in the population is divided by the total number of mitochondria assayed to provide an estimate of the percentage of nucleotides that have a mutation (a mutation inhibits PCR amplification). The segments with mutations can then be sequenced to determine the type of mutation present (transition or translation) (Chenna et al. 2003; Thompson et al. 2004; Goujon et al. 2010).

Using this method, Itsara et al. (2014) quantified the number and identity of mtDNA mutations in nervous and muscle tissues of young and old *Drosophila*. As predicted, mtDNA mutations were more numerous in old than young animals and mutations were more abundant in skeletal muscle than nervous tissue. Quantification of the types of mutations present revealed <10% of the mtDNA mutations in both young and old animals were associated with the transversion indicative of oxidative damage while more than 80% of the total mutations detected in young and old animal were the G:C to

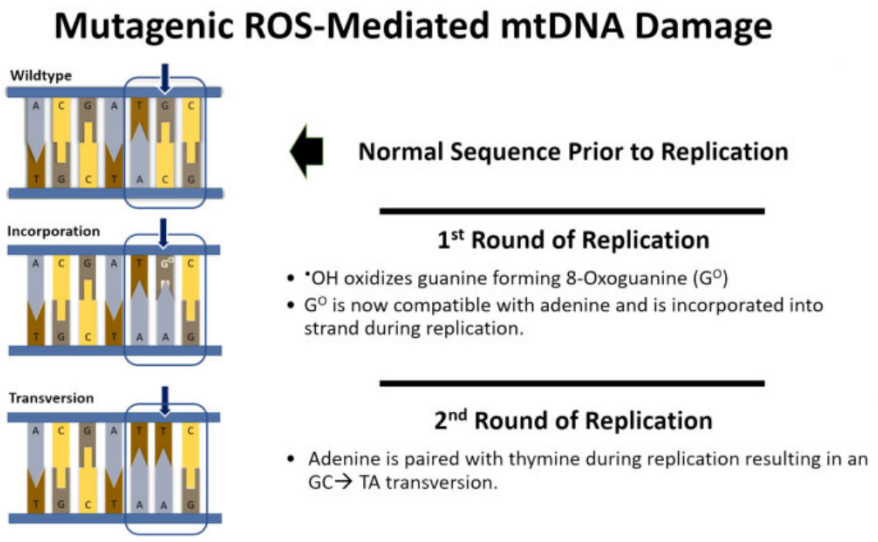

Fig. 4 Consequences of ROS damage to the mtDNA nucleotide guanine. Hydroxyl radical oxidizes guanine to form a nucleotide with the binding affinity of thymine. As a result, guanine is permanently changed to thymine during replication. This is described as a transversion mutation.

A:T transitions characteristic of replication error. These observations raise questions about the relative importance of oxidative damage to the accumulation of mtDNA mutations and mitochondrial dysfunction. The authors then assessed types of mutations found in flies with a reduced capacity to mitigate oxidative damage (superoxide dismutase 2, ie SOD2, mutants) relative to controls. The SOD2 mutants did not display an increase in G:C to T:A transversions, further suggesting oxidative stress has little impact on mtDNA mutation frequency. Similar patterns where transition mutations associated with replication error are substantially more numerous than replication-associated transversion mutations and where transition mutations increase with age have also been described in humans and mice (Vermulst et al. 2007; Kennedy et al. 2013). An important rarely considered caveat of these findings is that a transversion mutation is more likely to give rise to a non-synonymous mutation than a transition mutation (Yang and Bielawski 2000). Thus, even though ROS-induced mutations are substantially less numerous than replication error, they are more likely to cause a change in the amino acid sequence of a protein. The less abundant damage from ROS is more likely to cause dysfunction than replication error.

Replication error, senescence, and mitochondrial dysfunction

Novel experimental approaches have led to studies supporting the hypothesis of replication error playing a larger role than oxidative damage in the accumulation of mitochondrial mutations. In the early 2000s, researchers developed the POLG mouse, a strain of laboratory mouse carrying a homozygous knockout rendering it deficient in the nuclear-encoded catalytic subunit of mtDNA polymerase POLG (Trifunovic et al. 2004). This line of mice does not edit mtDNA replication errors and thus has an experimentally increased rate of replication error. Vermulst et al. (2007) confirmed that POLG mice carry many times more mtDNA transition mutations than wildtype mice. This increase in mtDNA mutation rate is associated with the early onset of phenotypes indicative of senescence, including alopecia (hair loss), kyphosis (dorsal curvature of the spine which hunches the back), and muscle atrophy. Furthermore, high levels of mutations are also associated with a reduction in the number OXPHOS complexes containing mtDNA-encoded subunits, including Complexes I, III, and IV (Hiona and Leeuwenburgh 2008; Edgar et al. 2009) as has been shown in the heart, liver, and skeletal muscle. Skeletal muscle of the POLG mouse also been shown to display impaired mitochondrial bioenergetics, lower maximum respiration rate when adenosine diphosphate and substrate are unlimited (i.e., state-3 respiration), lower ATP content, and decrease in mitochondrial membrane potential (Hiona and Leeuwenburgh 2008). In addition, POLG mice do not display a higher rate of ROS production or oxidative damage than wild-type mice (Kujoth et al. 2005; Trifunovic et al. 2005). Only small increases in ROS (H_2O_2) production were observed in end-stage animals (Logan et al. 2014).

The notion that disruption of the mtDNA replication machinery, which could be altered by errors

during replication, plays an important role in the decline in organ performance and aging has also been supported in other knockout and chimeric mice. For example, mitochondrial late-onset neurodegeneration (MILON) mice have a deficient transcription factor A, mitochondrial (TFAM); 60% of the neurons in homozygous carriers lack mitochondrial respiratory chains, whereas only 20% of the neurons lack mitochondrial respiratory chains in chimeric MILON mice (MILON × wildtype cross). Relative to wildtype mice which have neurons replete with fully functional mitochondria, the homozygous knockouts have the shortest lifespans and the lowest cognitive function, while the chimeras display intermediate values (Dufour et al. 2008). Thus, the assumption is that wild-type animals also accumulate errors during mitochondrial replication—as is indicated by the sequencing data described above. Furthermore, wild-type animals live-longer than POLG mice because they repair a proportion of the mitochondrial replication errors generated as new genomes are produced. Ultimately, senescence has been attributed to the effect that the natural accumulation of replication error-based mutations has on the replication machinery and on the mitochondrial proteins that function in the electron transport system (Larsson 2010; DeBalsi et al. 2017).

Mitochondrial mutations and longevity

Our understanding of the relationship between mitochondrial replication error and organismal senescence is largely based on studies that have employed genetic manipulations of the mitochondrial replisome. For these results to be applicable to natural populations, we would predict that replication errors increase with age and for the increase to ultimately reach a level which will contribute to mitochondrial dysfunction, a senescent phenotype, and ultimately, the death of the individual. Furthermore, we would also predict that replication error is positively correlated with rate of aging between species. The data that can be applied to these predictions in wild-type animals is equivocal.

Recent studies used very high-coverage sequencing to evaluate a larger proportion of the mitochondrial genome. Kennedy et al. (2013) compared the mtDNA mutational load of tissues collected from the brain of infants (<1 year) and elderly humans (79–90 year) with no known pathologies at the time of autopsy. They found transition mutations indicative of replication error were five times more abundant in the elderly sample than in the infant samples. Furthermore, they found transversion mutations, characteristic of ROS induced damage, do not increase with age. Ameur et al. (2011) quantified mutations in the full mitochondrial genome of the livers collected from inbred lab mice and found no change in the number of mutations due to transitions or transversions between 30- and 84-week old mice. Furthermore, Ma et al. (2018) also used high coverage sequencing to quantify mutations in the full mt genome for seven different somatic organs in wild-type mice and found no evidence of somatic mtDNA mutations in mice from 1 to 34 months of age. While there are several examples of studies which show increases in mtDNA mutations with age (Pikó et al. 1988; Itsara et al. 2014), it is clear that the magnitude of change is very low.

Before the excitement of first publication on the POLG mouse, Elson et al. (2001) modeled the accumulation of replication error in non-dividing human cells using available data on the typical number of mitochondria present in a cell, and estimated rates of replication and replication error. Despite the high turnover rate of the mitochondria, the authors concluded that only those cells that begin the accumulation of errors early in life and proliferate by genetic drift (clonal expansion) have the potential to accumulate enough error to alter mitochondrial performance (Elson et al. 2001). If this model is accurate, it would be unlikely that short-lived species would accumulate sufficient damage to mtDNA to shorten their lifespan (Kauppila et al. 2017). Yet to our knowledge, no direct comparisons have been made quantifying the rate of replication error between species.

Measuring replication error with sequencing methods is a challenging endeavor because PCR methods can introduce mutations and because genomic methods fragment nDNA and mtDNA molecules. As a result, genomic sequences are derived from a consensus of many aligned fragments. Because mutations to mtDNA are largely random, occurring at different locations in each of the thousands of plasmids that occur in each cell, it can be impossible to distinguish true mutations from erroneous mutations during sequence alignment (Larsson 2010). Ultra-depth sequencing overcomes some of these challenges. The more recent Maximum Depth Sequencing method (MDS) will likely helps investigators to remove PCR and alignment errors. MDS sequences the DNA fragment in both directions multiple times (Jee et al. 2016), allowing the investigator to distinguish amplification and sequencing errors from mutations (Hiatt et al. 2013; Jee et al. 2016; Simonsen et al. 2018; Sloan et al. 2018). While it is unclear whether Ma et al. (2018) did not detect somatic mutations due to

failure to recognize the mutations that were present, it is clear the mtDNA mutational load was low across tissues in these mice.

Finally, providing additional support for the idea that short-lived species, such as mice, do not accumulate sufficient replication error to stimulate senescence, Vermulst et al. (2007) compared the total mtDNA mutational load of full bred POLG mutants ($POLG^{mut/mut}$), heterozygous POLG mice ($POLG^{+/mut}$) that do not display the early onset of senescence found in full bred mutants ($POLG^{mut/mut}$), and wild-type mice ($POLG^{+/+}$). The total mtDNA mutational load of heterozygous POLG mice was found to be lower than full bred mutants, but was dramatically higher than age-matched wild-type mice and remained higher than old wild-type mice (2 years) (Vermulst et al. 2007). Given the mutational load of wild-type mice remains lower than heterozygous mice that do not display early senescence, the authors concluded the mutational load of wild-type lab mice does not reach a level that would contribute to a senescent phenotype. This study provides more evidence that wild-type mice are unlikely to reach an mtDNA mutation load which would hasten senescence, at least under the controlled conditions of the lab.

Application to wild populations

As evolutionary ecologists propose ever more detailed explanations for the evolution of life histories in natural systems (Zera and Harshman 2001; Flatt and Heyland 2011; Hood et al. 2018), there has arisen an increasing need to include cellular and biochemical mechanisms into ecological theory. For instance, ideas centered on energetic tradeoffs as the underlying reason for senescence only make sense if such tradeoffs are biologically realistic at both a whole-organism and cellular levels. Because organismal biologists often have limited training in cell and molecular biology, fully integrating new discoveries from cell and molecular biology laboratories into whole-organism investigations is challenging. But lines of research such as the relative importance of free radical damage versus replication error in the mutation of mtDNA and the decline of cell performance lie at the heart of evolutionary and ecological processes. The only way forward toward advancing basic understanding of life history evolution is through more integrative studies that keep pace with discoveries in cell and molecular labs.

Our understanding of the role of replication error in the accumulation of mtDNA mutations is based entirely on humans and laboratory animals maintained under constant environments devoid of natural stressors. None of the published empirical studies of replication error allowed non-human animals to breed, experience natural stressors, or engage in levels of activity that would be typical of animals in the wild. None of the gene-modification studies allowed animals to maintain natural social structures or experience variance in their diet, natural temperature and light fluctuation, nor were they exposed to pathogens, typical of most animals. These are not criticisms of the important and ground-breaking work done in these cell and molecular labs. Rather, the shortcoming of many foundational research programs in cell and molecular biology should be viewed as doors of opportunity for organismal biologists. Evolutionary ecologists have insights that are needed by molecular biologists as much as cell and molecular biologists have insights that are beneficial to ecologists.

As one example, in our studies of wild-derived mice, individuals can live for more than 3 years when held in the plastic box environment used in all laboratory mouse studies. When mice from the same wild-derived stock are held in semi-natural enclosures in which they must deal with change in ambient temperatures and compete with other mice for food, nests, and mates, their lifespans are reduced. We commonly observed mice in the semi-natural population displaying classic symptoms of senescence—alopecia, kyphosis, and muscle atrophy—at less than 2 years (W.R. Hood, personal observation). We know nothing about how natural activity, breeding, and other stressors may have impacted the rate of mitochondrial turnover and the accumulation replication error in these animals. We know that exercise can help rescue the early onset of senescence displayed by POLG mice (Safdar et al. 2011; Safdar et al. 2015). We know that peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α), the master regulator of mitochondrial biogenesis (Fernandez-Marcos and Auwerx 2011), increases during reproduction in wild-derived house mice and lab rats (Hyatt et al. 2017; Mowry et al. 2017). While an increase in PGC-1α is typically associated with improved bioenergetic capacity, an upregulation of PGC-1α can cause an increase in mtDNA mutations (Dillon et al. 2012). Perhaps a life of active breeding could result in a high rate of mitochondrial turnover. We also know replication error is highest and is most likely to contribute to senescence when it begins accumulating early in life (Lakshmanan et al. 2018).

Genomic data suggest that longevity covaries with mutation rate across species of mammals

(Nabholz et al. 2008; Welch et al. 2008) and perhaps other taxa (Hua et al. 2015). Given that replication error has been proposed to be more important than ROS-induced mtDNA mutations, it is critical that we consider the role of replication error in life-history evolution. We encourage field biologists to stay current on discoveries made in cell and molecular biology labs—including the potential role of mtDNA mutations arising from replication error or oxidative damage—but also to look for novel insights in their own study systems.

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