Mitochondrial Short-Term Plastic Responses and Long-Term Evolutionary Dynamics in Animal Species

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Abstract

How do species respond or adapt to environmental changes? The answer to this depends partly on mitochondrial epigenetics and genetics, new players in promoting adaptation to both short- and long-term environmental changes. In this review, we explore how mitochondrial epigenetics and genetics mechanisms, such as mtDNA methylation, mtDNA-derived noncoding RNAs, micropeptides, mtDNA mutations, and adaptations, can contribute to animal plasticity and adaptation. We also briefly discuss the challenges in assessing mtDNA adaptive evolution. In sum, this review covers new advances in the field of mitochondrial genomics, many of which are still controversial, and discusses processes still somewhat obscure, and some of which are still quite speculative and require further robust experimentation.

Key words: mitochondria, mitochondrial DNA, mitochondrial mutations, mitochondrial epigenetics, plasticity, adaptation.

Significance

Mitochondria are on the frontline of the cellular response to the environment and emerging data suggest that mitochondria can fuel phenotypic variation and evolutionary innovations. The primary aim of this paper is to review the advances in our understanding of how mitochondrial epigenetics and genetics can contribute to animal plasticity and adaptation. The studies summarized and questions posited in this review paper have the potential for transforming our understanding of the role of mitochondria in metazoans.

Introduction

In the light of global warming and climate change, it is becoming of general interest to understand the mechanisms by which animal species face changing environments. Animals respond to environmental factors over time in different ways: 1) for motile species by migrating to more favorable conditions but also 2) through plasticity, namely short-term changes during their lifetime (e.g., epigenetic modifications and gene expression changes) and 3) through adaptation, namely long-term changes across generations, that is, heritable evolutionary responses, resulting in genetically distinct populations—potentially even new species. Genetic diversity thus fundamentally serves as a way for animal populations to adapt to changing environments, and mutation is the ultimate mechanism generating genetic variability.

Although empirical examples of rapid responses and evolutionary adaptations involving nuclear epigenetic (e.g., in the form of DNA methylation and noncoding RNAs) and genetic mechanisms (e.g., through the evolution of lineage-specific or adaptive mutations and genes) exist from a range of animal species (Smith and Eyre-Walker 2002; Bamshad and Wooding 2003; Jaenisch and Bird 2003; Khalturin et al. 2009; Smith et al. 2016; Cavalli and Heard 2019), the importance of mitochondria and their genomes (mtDNAs) in promoting adaptation to both short- and long-term environmental changes using the same mechanisms remain largely unexplored (e.g., Breton et al. 2014; James et al. 2016; Riggs et al. 2019). Experimental evolution studies have started to test capacity for mtDNA variation to respond to selection (e.g., Lajbner et al. 2018; Immonen et al. 2020) but such research is still

in its infancy. This major knowledge gap is surprising given the pivotal role of mitochondria in cell survival and functions, ageing, and human health.

In this article, we review the advances in our understanding of the mitochondrial epigenetics and genetics mechanisms that allow animals to survive in a changing world. There have been excellent recent reviews on the evolutionary relevance of mitonuclear interactions and coevolution contributing to the dynamics of mtDNA regulation and evolution (e.g., Matilainen et al. 2017; Sloan et al. 2018; Mottis et al. 2019; Hill et al. 2019; Hill 2020), and many processes overviewed in this paper might be moderated by mitonuclear interactions. Here, we focus on the diversity of forms of regulation from within the mitochondria, that is, the proximate mechanisms involved in short-term mitochondrial plastic responses, and also on long-term mitochondrial evolutionary dynamics, that is, mtDNA mutations and adaptations. Specifically, the paper contains two main sections in which we respectively discuss aspects of 1) how mtDNA expression and mitochondrial function are internally regulated via mtDNA methylation, mtDNA-derived noncoding RNAs and micropeptides, and 2) the sources of mtDNA mutations, mtDNA adaptations as well as the challenges we face in assessing mtDNA adaptive evolution due to the complexity of mitochondrial biology. Many of these aspects are still controversial and need to be further clarified both experimentally and theoretically.

Mitochondrial Plasticity: Short-Term Response of Animals to Changing Environments

Mitochondrial plasticity refers to mitochondrial adjustments to different environmental cues and metabolic alterations to meet the bioenergetic demands of the cell. One main mechanism by which animal mitochondria may respond to acute or longer term environmental changes, such as seasonal variation, is by modulating gene expression. In mammals, for example, in altered thermal environments, transcriptional regulation of mitochondrial capacities implicates thermal sensing proteins, which provide a thermosensory input to the hypothalamus, which in turn modulates the expression of the transcriptional coactivator 1-alpha (PGC-1α). PGC-1α is a nuclear-encoded protein that interacts with the nuclear respiratory factors NRF1 and NRF2 that control transcription of nuclear and mitochondrial genes involved in mitochondrial respiratory function, including the mitochondrial transcription factor TFAM for the regulation of mtDNA replication and expression (reviewed in Seebacher et al. 2010). This example illustrates well the crosstalk between the nucleus and the mitochondria leading to the maintenance of cellular health and homeostasis. However, nucleus-to-mitochondria (anterograde) and mitochondria-to-nucleus (retrograde) genetic regulation alone cannot fully explain mitochondrial plasticity. There are other processes that regulate mitochondrial DNA replication and gene expression within the mitochondrion, such as mitochondrial epigenetic mechanisms, and there are also mtDNA-encoded factors that modify cellular metabolism not necessarily always through "communication" with the nucleus. In this section, we will briefly review some of these factors underlying mitochondrial plasticity from a "strictly mitochondrial genome point of view," that is, those directly acting on or encoded by the mtDNA that regulate mitochondrial gene expression and function, including mtDNA methylation, mtDNA-derived noncoding RNAs and micropeptides. Other mechanisms, such as posttranslational modifications of mitochondrial nucleoid proteins or mtDNA-encoded proteins (reviewed in Stram and Payne 2016; Sharma et al. 2019), posttranscriptional modifications of mtRNA (reviewed in Pearse et al. 2017), as well as mitochondrial fatty acid composition, mitochondrial fusion and fission, and ROS signaling, which are also important features for maintaining mitochondrial plasticity (e.g., Seebacher et al. 2010; Bahat and Gross 2019) will not be discussed. Moreover, we will focus on mechanisms that seem to be conserved among animal taxa, so that we do not attempt to exhaustively review all exceptions. For example, emerging evidence suggests that RNA editing could be used for acclimation or acclimatization (reviewed in Rosenthal 2015). To our knowledge however, mitochondrial RNA editing, even if it has evolved multiple times independently (Chateigner-Boutin and Small 2011), does not seem to be widespread in metazoans and its role in mitochondrial plasticity still remain obscure.

The Controversial History of mtDNA Methylation

Epigenetics refers to the mechanisms allowing the modification of gene expression without directly altering the nucleotide sequence (see Cavalli and Heard 2019 for a recent review). In other words, epigenetic mechanisms allow for the generation of phenotypic variations in organisms, while keeping the genome intact (Leung et al. 2016; Cavalli and Heard 2019). Both nuclear and mitochondrial DNAs can be regulated via epigenetic mechanisms, but the understanding of mitochondrial epigenetics is still in its infancy. One interesting example is DNA methylation, which is the most studied epigenetic mechanism. In animals, methylation of nuclear DNA occurs mainly on a cytosine that precedes a guanine, at its fifth carbon (5-methylcytosine or 5mC). One such dinucleotide sequence is called "CpG" and can be found in large quantities in certain regions of the nuclear genome, forming CpG islands (CGI) (Illingworth and Bird 2009). Methylation is found mainly in promoters, where it represses expression of the associated gene, for example, by preventing the binding of transcription factors, thereby blocking its expression (Illingworth and Bird 2009).

In contrast to the nuclear DNA, the presence and role of cytosine methylation in the mitochondrial DNA have been a matter of debate since the 1970s (reviewed in van der Wijst and Rots 2015; Castegna et al. 2015; Lambertini and Byun 2016; D'Aquila et al. 2017; Mposhi et al. 2017; Coppedè and Stoccoro 2019; Sharma et al. 2019; Leroux et al. 2021). On the one hand, some recent studies suggest that this process is virtually absent or very rare in mammals (e.g., Liu et al. 2016; Mechta et al. 2017; Matsuda et al. 2018; Owa et al. 2018), thus questioning its functional relevance. For example, Matsuda et al. (2018) exhaustively analyzed mouse mtDNA using three methods that are based upon different principles for detecting methylated cytosines, that is, whole genome bisulfite sequencing (WGBS), treatment with methylated cytosine-sensitive endonuclease McrBC and mass spectrometric nucleoside analyses of highly purified mtDNA preparations, and detected very low levels of 5-methylcytosines (<2%), questioning the putative role of methylation in the regulation of mtDNA gene expression.

On the other hand, a growing body of literature suggests that methylation adds an epigenetic layer of regulation control of mtDNA replication and transcription (D'Aquila et al. 2017 and reviews aforementioned). Several studies reported significant correlations between mtDNA epigenetic marks (i.e., 5-methylcytosines as well as 5-hydroxymethylcytosines), dietary, pharmacological agents, exposure to environmental pollutants and peculiar phenotypes, ageing, and diseases (D'Aquila et al. 2017 and reviews aforementioned). The majority of these studies assessed mtDNA methylation levels of the D-loop region, which contains essential replication and transcription elements. For example, a study by Liao et al. (2016) showed that with respect to the control group, fish fed a high-lipid diet were characterized by an increase of D-loop methylation. Coupled to these studies were the discoveries of DNA methyltransferases targeted to and functioning in human and mouse mitochondria, modifying mtDNA methylation levels and presumably influencing mitochondrial transcription (Chestnut et al. 2011; Shock et al. 2011; Wong et al. 2013; Dou et al. 2019; Patil et al. 2019). However, despite all these observations, mtDNA methylation remains a matter of debate due to contradictory reports from WGBS studies, but recent work suggests that this controversy can in part be attributed to methodological considerations.

Indeed, by taking methodological adaptations for investigating mtDNA methylation via WGBS (e.g., accounting for or avoid nuclear contamination and linearize the molecule prior to bisulfite conversion to avoid secondary structure effects), two recent and independent studies, respectively on human cells (Patil et al. 2019) and cow oocytes and blastocytes (Sirard 2019), demonstrated that the mtDNA is extensively methylated (>10%), with a concomitant decrease in gene expression being observed, thus challenging the notion that mtDNA methylation is not biologically relevant. These two studies (Patil et al. 2019; Sirard 2019) also revealed L-strand and non-CpG methylation biases in mammalian mtDNAs, supporting the suggestion by Dou et al. (2019) that most WGBS studies focused on only methylated CpGs and did not perform strand-specific analysis, and this, that is, neglecting non-CpGs and combining reads of both strands for mapping methylation, probably misled to previous conclusions of the absence of global mtDNA methylation. For example, the L strand in the human mtDNA possesses more than twofold C sites than the H strand, and among all C sites ($N = 7{,}350$), CpGs are underrepresented ($N = 435$). This could partly explain why mtDNA methylation mainly occurs on the L strand. In addition, because most protein-coding genes (12 out of 13) use L strand as template, the regulation of the L strand by methylation may relate to mtDNA gene expression regulation (Dou et al. 2019).

That being said, by reanalyzing previous WGBS data, Dou et al. (2019) validated that mitochondrial genomes in humans, mice, and zebrafish are strongly biased to L-strand non-CpG methylation with conserved peaks (>10% methylation) at gene–gene boundaries, and conserved methylation patterns across different species and developmental stages. Moreover, knockout of de novo methyltransferase *DNMT3A* perturbed mtDNA methylation patterns (but not global levels) and altered mitochondrial gene expression, copy number, and oxygen respiration (Dou et al. 2019). Similar results, that is, knockdown of DNA methyltransferase enzymes affecting methylation levels, were reported by Patil et al. (2019), who also suggested the possibility of fine, transient, and gene-specific transcriptional regulation by methylation to meet the energy demand of cells. This hypothesis was supported by the observation that the genes of a certain promoter region of the D-loop were differently affected in the absence of the methyltransferase *DNMT3B* (Patil et al. 2019), suggesting the existence of a gene-specific regulation. Similarly, Sirard (2019) reported an association between a low level of methylation and a higher expression of certain protein-coding genes, and conversely, a high level of 5mC in less expressed genes. Collectively, these data provide strong support for functional relevance of mtDNA methylation in animals.

Interestingly, two recent researches suggested that adenine methylation is also an important epigenetic mechanism regulating mitochondrial function (Koh et al. 2018; Hao et al. 2020). Koh et al. (2018) first reported the presence of N6-methyldesoxyadenosine (6 mA) in humans and showed that 6 mA levels in mtDNA are much higher than in nuclear DNA, and that adenine demethylation causes a decrease in ATP production. Similarly, Hao et al. (2020) showed that mammalian mtDNA is enriched for 6 mA compared with nuclear DNA, and that the activity of the methyltransferase that mediates mtDNA 6 mA methylation (Methyltransferase Like 4 or *METTL4*) can decrease mtDNA transcription and copy number. Moreover, 6 mA levels in mtDNA could be further elevated under hypoxia, suggesting regulatory roles for 6 mA in mitochondrial stress response (Hao et al. 2020).

Despite the growing body of literature on mtDNA methylation, its role and effects in animal species still remain largely obscure (e.g., Kowal et al. 2020), probably because of previous conflicting reports surrounding cytosine methylation, and also because most articles published to date focused on human and mouse mtDNAs (e.g., Bellizzi et al. 2013; Lambertini and Byun 2016; D'Aquila et al. 2017; Matsuda et al. 2018; Patil et al. 2019). Mitochondrial DNA methylation seems to affect mitochondrial gene expression and function, and thus likely represents an additional key mechanism by which animals could respond quickly (in real time) to environmental change (e.g., Bartelli et al. 2018). Moreover, mtDNA methylation seems to be affected by numerous factors and could thus represent a useful biomarker for harmful environmental and nutritional factors (Iacobazzi et al. 2013), and also for disease detection and diagnosis (Gao et al. 2017). Further studies are needed to add more precision and clearer understanding of the phenomenon.

Intramitochondrial Actions of mtDNA-Derived Noncoding RNAs

Epigenetic regulation of mitochondrial gene expression can also be accomplished by noncoding RNAs (ncRNAs). An ncRNA is a functional molecule transcribed from DNA but not translated into a protein, the most well-known being the tRNAs and rRNAs involved in protein synthesis. Mitochondrial epigenetic-related ncRNAs, that is, long noncoding RNAs of >200 bp (lncRNAs) and small noncoding RNAs, can be of nuclear or mitochondrial origin. They generally regulate gene expression at the transcriptional and posttranscriptional levels. One recent example is *Cerox1*, a nuclear lncRNA conserved across placental mammals that has been shown to modulate mitochondrial complex I subunit transcripts, increasing complex I subunit protein abundance and enzymatic activity, and decreasing ROS production (Sirey et al. 2019). In this article, however, we will not discuss ncRNAs produced by the nuclear genome and imported into mitochondria (nor ncRNAs produced by mtDNA and acting as retrograde signaling molecules), since the subject has been recently reviewed by others (e.g., Dong et al. 2017; Vendramin et al. 2017; Zhao et al. 2018; Jeandard et al. 2019; Cavalcante et al. 2020; Gusic and Prokisch 2020). We will rather provide a brief overview of mitochondrial epigenetic-related ncRNAs transcribed from the mtDNA and acting inside mitochondria, although their mode of action in mitochondria still remains mostly enigmatic.

Indeed, the mitochondrial genome is known to produce a set of lncRNAs (mtlncRNAs; Dong et al. 2017; Zhao et al. 2018; Sharma et al. 2019 for reviews) as well as small noncoding RNAs (microRNAs located in other typical mitochondrial genes; Geiger and Dalgaard 2017; Pozzi and Dowling 2019; Sharma et al. 2019; Pozzi and Dowling 2020; Pozzi and Dowling 2021) that could participate in the regulation of mitochondrial gene expression. mtlncRNAs with putative intramitochondrial functions include the simple antisense transcripts *lncND5*, *lncND6*, and *lncCytb*, which were first identified by strand-specific RNA-seq of purified mitochondria and by RT–qPCR (Mercer et al. 2011; Rackham et al. 2011). These mtlncRNAs have been shown to create RNA–RNA duplexes with their complementary mRNAs, supporting the idea that they could regulate mRNA expression and stability (Rackham et al. 2011). Two other mtlncRNAs that are thought to play a role in the regulation of mitochondrial gene expression are *MDL1* (Mitochondrial D-Loop 1), which mostly covers the entire mitochondrial D-loop region of the human mtDNA, and *MDL1AS* (*MDL1* Anti Sense), which is the antisense transcript of *MDL1* (Gao et al. 2018). *MDL1* and *MDL1AS* were proposed to be precursors of mitochondrial transcription initiation RNAs (Gao et al. 2018).

Contrary to mtlncRNAs, which have been mainly studied in human and mice, putative mitochondrially produced microRNAs (i.e., from ~15nt to ~120nt) have been described in a variety of animal species (e.g., Mercer et al. 2011; Ro et al. 2013; Bottje et al. 2017; Pozzi et al. 2017; Riggs et al. 2019; Pozzi and Dowling 2019; Passamonti et al. 2020; Pozzi and Dowling 2020). Their mitochondrial origin has been demonstrated in various ways, for example 1) they are not expressed in Rho0 cells devoid of mitochondrial DNA (Ro et al. 2013), 2) they map exclusively to the mtDNA sequence even in species with high NUMT levels (mitochondrial pseudogenes in the nuclear genome), and 3) their tissue-specific abundances are strongly associated with the mtDNA content (Pozzi and Dowling 2019). The historical nomenclature for these mitochondrial microRNAs is a bit confusing (see Pozzi and Dowling 2020). The term mitomiRs refers to microRNAs of nuclear or mitochondrial origin identified in the mitochondria (Bandiera et al. 2011; Duarte et al. 2014), whereas the term mt-miRNAs refers to microRNAs that are specifically encoded by the mtDNA and able to bind Argonaute protein or *AGO2*, a key element involved in RNA interference (RNAi, see below) (Pozzi and Dowling 2020). Several groups have reported that mitomiRs of mitochondrial origin can regulate the expression of mitochondrial genes, and to our knowledge, many of them appear to do it in a RNAi-dependent manner (e.g., Ro et al. 2013; Sharma et al. 2019; Cavalcante et al. 2020; Pozzi and Dowling 2020).

By directly targeting mRNAs, miRNAs represent one important class of posttranscriptional regulators of gene expression. Specifically, a mature miRNA associates with a partially complementary regulatory region of a target mRNA as well as with *AGO2*, an endonuclease shared across multiple species and involved in RNAi (Ha and Kim 2014; Cloonan 2015; Pozzi et al. 2017). With other partners, they form a cytoplasmic ribonucleoprotein RISC complex that is able to hinder the binding of the target mRNA to the ribosome (Cloonan 2015). However, it is still unclear whether such a mechanism operates inside mitochondria

or if there is an alternative RNAi-like machinery operating inside the organelle (Ro et al. 2013; Cavalcante et al. 2020; Pozzi and Dowling 2020). The latter idea is supported by the observations that key proteins involved in RNAi, such as *AGO2* and *DICER*, were absent in highly enriched mitochondrial fractions from human HEK293T cells, suggesting that mitochondria lack the canonical miRNAs biogenesis machinery (Ro et al. 2013). In sharp contrast, strong evidence that *AGO2* and other key components of the canonical RNAi machinery may function in mitochondria were provided by different studies (e.g., Bandiera et al. 2011; Zhang et al. 2014; Geiger and Dalgaard 2017). For example, a mitochondrial localization of *AGO2* was reported following mitochondrial immuno-isolation after differential centrifugation to wash the organelles in stringent conditions, leading to highly purified mitochondrial fractions (Bandiera et al. 2011). Additional results and interpretations of Bandiera et al. (2011) were rather convincing, such as 1) the use of four prediction programs to identify subcellular protein localization (i.e., TargetP, MitoProt II, Predotar, and ESLPred), which all consistently predicted a mitochondrial localization of *AGO2*, 2) the binding of *AGO2* to some mitochondrial transcripts such as *COX3*, and 3) the insights from other proteomic studies of *AGO2* partners, which identified mitochondrial proteins mostly from the inner membrane, including many ATP/ADP translocases, carriers and ribosomal proteins as binding partners (Höck et al. 2007). All together, these observations suggest that putative mt-miRNAs could inhibit mitochondrial mRNA translation in a RNAi-dependent manner, although canonical RISC-activity in mitochondria still needs to be clearly demonstrated. It is also possible that small noncoding mtRNAs do not act exclusively through RNAi, as some of them seem to enhance the production of their host mitochondrial genes (e.g., Ro et al. 2013; Sharma et al. 2019), and/or act as regulators through interactions with different proteins (e.g., Geiger and Dalgaard 2017).

Recent research highlights regulation via small noncoding mtRNAs as an emerging mechanism by which animals can adjust the expression of their mitochondrial genome in relation to cellular conditions and energetic demands. For example, a study by Riggs et al. (2019) provided evidence that small mtDNA-encoded RNAs may play a role in supporting anoxia tolerance in embryos of the annual killifish *Austrofundulus limnaeus*. It is now clear that the mtDNA harbors multiple gene layers, with different mitochondrial products encoded within multiple genes, such as noncoding RNAs and micropeptides, which are discussed below, and the question arises whether the presence of overlapping selection pressures could alter the strength or direction of selection on particular regions of mtDNA sequence. As suggested by Pozzi and Dowling (2020), the presence of "genes within genes" should increase the effect of purifying selection in order to preserve the function of these products. Comparative studies across a growing number of species will be crucial to fully test this hypothesis.

Mitochondrially Encoded Micropeptides That Modify Cell Metabolism

For many years, most scientists assumed that, in eukaryotes, each mature nuclear mRNA encodes a single functional open reading frame (ORF), but recent findings have revealed in humans and other organisms, that many mRNAs encode more than one protein, that is, they code for a large protein and one or several small proteins (Samandi et al. 2017). These findings indicate that the size and complexity of most eukaryotic nuclear proteomes have probably been greatly underestimated and this is also true for mitochondrial proteomes, including in humans (Capt et al. 2016; Angers et al. 2019; Samandi et al. 2017; Miller et al. 2020). This is supported by recent discovery of small proteins of functional importance, for instance *Humanin*, *MOTS-c*, and *SHLPs* (small humanin-like peptides), that is, micropeptides 16–38 amino acids long that are encoded within the 16S (*Humanin* and *SHLPs*) and the 12S rRNA (*MOTS-c*) genes in the human mtDNA (Lee et al. 2015; Cobb et al. 2016; Kim et al. 2018). Of these, *MOTs-c*, which is involved in metabolic regulation, appears to exert its function in the cytoplasm (Lee et al. 2015), and *Humanin*, the first discovered mtDNA-encoded micropeptide, to modulate mitochondrial biology, cell proliferation, and cell survival through receptors located outside mitochondria (reviewed in Miller et al. 2020). Of the six *SHLPs*, *SHLP2*, and *SHLP3* have been shown to increase mitochondrial oxygen consumption rate and ATP levels while reducing reactive oxygen species, indicating them as mitochondrial modulators (Cobb et al. 2016). However, this was observed through an incubation of cells with *SHLP2* and *SHLP3*, that is, by testing their exogenous effects on mitochondria (Cobb et al. 2016). It thus remains to be established if mtDNA-encoded micropeptides exert their or some of their functions in the mitochondrial compartment. On a final note, the presence of *Humanin* and *SHLP6* was also detected through comparative genomic analyses in birds (Mortz et al. 2020), suggesting the possibility that the mitochondrial rRNA genes may encode small bioactive peptides in a variety of animal taxa.

Mitochondrial Adaptation: Long-Term Response to Changing Environments

Evolution can be defined as the change in allele frequencies through time. Such change can be due to chance events (mutation-drift), or the result of differences in fitness. Interestingly, the sources of mitochondrial genome mutations are still a matter of debate. In animals, there is a wide consensus that mtDNA typically mutates faster than nuclear DNA (Ballard and Whitlock 2004) and it has long been presumed that this higher mutation rate was

predominantly attributable to reactive oxygen species (ROS) produced in mitochondria during respiration (Harman 1972; Miquel et al. 1980; Richter et al. 1988; Shigenaga et al. 1994). This assumption has however been challenged by findings suggesting that mutations are mostly an endogenous property of mtDNA replication and repair machinery (Milani and Ghiselli 2015; Melvin and Ballard 2017; Hood et al. 2019). Indeed, several studies have questioned whether mutagens necessarily lead to extensive DNA damage, including ROS (Szczepanowska and Trifunovic 2015; Wanagat et al. 2015). This rethinking was due to several considerations, among which 1) the resilience of mtDNA to many mutagens of the nuclear DNA (nDNA) (Valente et al. 2016), 2) the mtDNA protection in the nucleoid—forming nucleoid–protein–DNA structures in the inner mitochondrial membrane—(Kucej and Butow 2007) that shields the mtDNA from mutagens by packaging it as chromatin does for the nDNA, and 3) the rapid scavenging of ROS, that actually minimizes their damaging potential (Sheng et al. 2014; Melvin and Ballard 2017).

It is becoming evident that the majority of mtDNA mutations probably derive from errors during mtDNA replication without adequate repair mechanisms (Zheng et al. 2006; Szczepanowska and Trifunovic 2015; Kauppila et al. 2017). If the origin of mtDNA mutations was oxidative stress, the most common change would be mis-incorporation of an A base, resulting in a G: C to T: A (G → T) (Bohr 2002). However, recent analyses suggested that G: C to A: T transitions (C → T) are the most common mtDNA mutations (Khrapko et al. 1997; Zheng et al. 2006; Lawless et al. 2020). According to these studies, this type of change is most likely a consequence of errors due to Pol-γ activity, the enzyme responsible for replication of the mtDNA (Zheng et al. 2006; Melvin and Ballard 2017), thus minimizing the role of ROS in mtDNA mutagenesis. That being said, oxidative damage to Pol-γ may also cause reduced replication fidelity (Anderson et al. 2020), and the fidelity of mtDNA replication does not depend only on Pol-γ but also on other molecules and factors involved in mtDNA maintenance and repair, as well as on local DNA sequence environment (Szczepanowska and Trifunovic 2015). One of the compelling lines of evidence against the historical view that high animal mtDNA mutation rates are simply the result of oxidative damage associated with mitochondrial function is the fact that some other eukaryotic lineages (like plants) have very low mitochondrial mutation rates (Wolfe et al. 1987), and that these low mutation rates are dependent on specialized repair machinery that is actually not present in animal systems (Wu et al. 2020).

The change in allele frequencies due to differential fitness is caused by natural selection and it can be either a decrease in the frequency of deleterious alleles (defined as negative or purifying selection), or an increase in frequency of beneficial alleles (positive selection). Natural selection is responsible for adaptation, and it can be the result of a direct influence of a genetic variant on a phenotype (direct selection), or of selection acting at a linked locus (indirect selection). For a long time, the textbook notion was that the mtDNA evolves mainly under purifying selection, and that the genetic variance that we observe in populations is neutral (Ballard and Whitlock 2004). Given the central role of mitochondria in eukaryotic life, it is reasonable to think that the mtDNA influences several traits and phenotypes, thus being involved in adaptive processes. Indeed, there is increasing evidence of mtDNA genetic variance being linked to phenotypic variance across several traits (Dowling 2014), and that mitochondria undergo a significant amount of adaptive evolution (James et al. 2016). Some examples of recent works reporting evidence of adaptive mtDNA evolution are included in table 1. The traits influenced by mtDNA showing signs of adaptation are predictably broad, the most investigated being thermal/altitude adaptation, longevity, effects of diet on metabolism, stress tolerance, and reproduction. Most of the evidence of adaptive evolution, though, is based on correlations and manipulative experiments are still limited and restricted to a handful model species (Dowling 2014, Milani and Ghiselli 2020).

Experimental evolution studies are promising to test the idea that standing mtDNA variants can be shaped by selection imposed by environmental heterogeneity, and to understand mtDNA contribution to trajectories of adaptive evolution. In recent studies—for example, in *Drosophila* and *Callosobruchus* seed beetle (table 1)—environmental selection (e.g., replicated populations assigned to two divergent temperatures) was applied, then the changes in frequencies of mtDNA haplogroups across generations in each treatment were assessed. This can be a powerful way to test for a potential adaptive role of mtDNA.

Challenges in Assessing mtDNA Adaptive Evolution

Mitochondrial DNA mutation rate depends on life history of organisms (e.g., physiology, reproduction), so it is not surprising to see a large variance across different taxa (see Allio et al. 2017 and references therein). Interestingly, James et al. (2016) suggested that mitochondrial adaptive evolution is limited by the supply of mutations; a consequence of these observations is that organisms with different life histories and biology will have different adaptation potentials. For this reason, it is very important to estimate mtDNA mutation rates to infer evolutionary patterns, but the presence of multiple copies per organelle and multiple organelles per cell is a major source of complexity (reviewed in Schaack et al. 2020). Another consequence of mtDNA being present in multiple copies in each cell is that different variants can be present in the same organelle and/or cell, a condition defined as heteroplasmy. Once thought to be rare and not particularly relevant, now heteroplasmy is known to be widespread (Dowling 2014). An interesting feature of heteroplasmy is

Table 1
Recently Published Works Reporting Adaptive Evolution of Animal mtDNA

Adaptation	Investigated Organism(s)	References
Altitude/hypoxia	Mice, grasshoppers	Cheviron et al. (2014); Li et al. (2018)
Bioelectrogenesis	Electric fish	Elbassiouny et al. (2020)
Depth	Fish, scale worms, sea cucumbers, bivalves	Shen et al. (2019); Zhang, Sun et al. (2018); Mu et al. (2018); Yang et al. (2019)
Diet/metabolism	Vampire bats, fruit flies, ladybirds	Botero-Castro et al. (2018); Camus et al. (2017); Mossman et al. (2016); Yuan et al. (2020)
Evolution of soft shell	Turtles	Escalona et al. (2017)
Sexual dimorphism/antagonism	Fruit flies, mammals, seed beetles	Camus et al. (2017); Havird and McConie (2019); Immonen et al. (2020); Nagarajan-Radha et al. (2020)
Sperm competition	Pseudoscorpions	Padua et al. (2014)
Thermal adaptation	Fruit flies, mice, crabs, mammals, insects, seed beetles, European anchovies, planthoppers; birds	Camus et al. (2017); Cheviron et al. (2014); Chung et al. (2017); Frigault et al. (2017); Immonen et al. (2020); Lajbner et al. (2018); Silva et al. (2014); Sun et al. (2019); Lamb et al. (2018)

NOTE.—For the bibliographic research, we used the following criteria: 1) papers published in the last 5 years, 2) molecular/genomic approach (i.e., papers must include sequence analyses), and 3) papers must report specific adaptations, not just signatures of putative positive selection.

that the expression of a particular mitochondrial variant depends on its abundance in the mitochondrial population. There is a "threshold effect" by which a mtDNA variant will affect the phenotype only if it exceeds a certain frequency in the mitochondrial population, otherwise its effects are buffered by the more common variant (Ghiselli et al. 2013; Dowling 2014; Milani and Ghiselli 2015, van den Ameele et al. 2020). The segregation dynamics of mtDNA across organelles, cells, tissues, and generations will influence the distribution of variants, changing their frequencies and affecting their penetrance. Therefore, a variant can have different effects in different cells/tissues as heteroplasmy changes over time because of the partitioning of mtDNA into daughter cells at each cell division (van den Ameele et al. 2020; Zhang, Burr et al. 2018). The distribution of mtDNA variants is also affected by mitochondrial fission–fusion and intercellular transfers that make the situation even more dynamic (Busch et al. 2014; Sinha et al. 2016; Torralba et al. 2016). Since the copy number of a variant determines its expression, the changes in frequencies inside an organelle, a cell, a tissue, or a whole organism will affect the effective population size of that variant, with consequences on genetic drift and selection. Each time an mtDNA population goes through a reduction in copy number (genetic bottleneck)—for example, during mitochondrial fission, cell division, and gametogenesis—the relative frequencies of different variants change. The maintenance and distribution of different alleles in the mitochondrial population were initially thought to be governed exclusively by random genetic drift, but there is increasing evidence of selection, especially of the purifying type (Milani and Ghiselli 2015; Milani 2015; Zhang, Burr et al. 2018), but also selfish selection appears to be involved (see below). Interestingly, a reduction in mtDNA copy number per organelle/cell can expose low-frequency variants to natural selection when their abundance cross the threshold and they express their phenotype. Accordingly, the mechanism of mitochondrial fission can isolate deleterious variants that are then eliminated by mitophagy (Busch et al. 2014), and the reduction of mtDNA copy number per cell in gametes and during embryo development can increase the effectiveness of natural selection (Ghiselli et al. 2013; Milani and Ghiselli 2015).

Another consequence of mtDNA being present in multiple copies, is multilevel selection (Rand 2001), a condition that can lead to genomic conflicts, favoring the spread of selfish variants (see Phillips et al. 2015; Lindholm and Price 2016; Ma and O'Farrell 2016; Klucnika and Ma 2019). Uniparental inheritance of mitochondria reduces within-individual variation, in turn reducing within-individual selection, and this is often argued to be one of the main reasons behind its evolutionary success. However, uniparental inheritance favors the emergence of cytonuclear conflicts over sex ratio, sex determination, and sexual antagonistic variants (Unckless and Herren 2009; Dowling and Adrian 2019). Since mtDNA is usually maternally inherited, the evolutionary response to selection on mtDNA in males is greatly limited and this means that male-harming mtDNA variants can accumulate under mutation-selection balance (Frank and Hurst 1996). This principle was named "Mother's Curse" by Gemmell et al. (2004), and the situation just described has been referred to as a "weak form" of Mother's Curse (Dowling and Adrian 2019; Havird, Forsythe, et al. 2019). The "strong form" of Mother's Curse predicts that female-beneficial but male-harming variants can accumulate as a consequence of positive selection on sexually antagonistic mutations (Dowling and Adrian 2019; Havird, Forsythe, et al. 2019). Results of some empirical works are consistent with the Mother's Curse hypothesis (Rand 2001; Sackton et al. 2003; Camus et al. 2017; Milot et al. 2017; Vaught and Dowling 2018), but in some other cases no supporting evidence was found (Mossman

et al. 2016, 2017; Eyre-Walker 2017), so the generality of the phenomenon and its relevance to natural populations are still under study (Dowling and Adrian 2019; Rand and Mossman 2020). It has been shown that the effects of Mother's Curse can be curtailed by inbreeding or assortative mating allowing nuclear modifiers to be effective despite mtDNA not being transmitted by males (Unckless and Herren 2009; Wade and Brandvain 2009; Hedrick 2012), and that the context-specific nature of mitonuclear epistatic interactions (more below) could increase or minimize Mother's Curse impact (Rand and Mossman 2020). Interestingly, a theoretical model (Wade and Brandvain 2009) and experiments on *Drosophila* (Keaney et al 2020a, 2020b) revealed the possibility that also kin selection might allow mitochondria to respond to selection on both male viability and fertility. Clearly, the asymmetry between sexes concerning mitochondrial biology adds multiple layers of complexity to the study of adaptive evolution of mtDNA.

One last challenge we want to briefly mention here is mitonuclear coevolution. One of the consequences of the endosymbiotic origin of mitochondria and the retention of an mtDNA semi-independent from the nuclear genome, is that the two genomes have to coevolve, because fundamental cellular functions—such as energy production through oxidative phosphorylation—depend on the integration and interaction of molecules produced by either genome. So, a specific phenotype can be the result of the product of epistatic interactions—either conflicting or cooperative—among nuclear and mitochondrial genes by the environment, making the reconstruction of evolutionary patterns very difficult (Rand and Mossman, 2020). This also means that a mitochondrial variant can have different effects depending on the nuclear background, and that mitonuclear interactions and coevolution are heavily involved in the mechanisms of adaptation (Hill et al. 2019; Hill 2019). Almost all the processes overviewed in this paper might be moderated by mitonuclear interactions, and there is increasing evidence that such interactions are evolutionarily relevant (e.g., Rand et al. 2004; Bar-Yaacov et al. 2012; Osada and Akashi 2012; Barreto and Burton 2013; Gershoni et al. 2014; Sloan et al. 2018; Adrion et al. 2016; Havird and Sloan 2016; Yang et al. 2019; Piccinini et al. 2021). Mitonuclear interactions and coevolution is a quite complex topic, so an in-depth discussion would require a dedicated paper, rather than just a paragraph. Lately, this evolutionary mechanism has gained much attention from evolutionary biologists and some thorough reviews are available (Sloan et al. 2018; Hill 2019; Hill 2020).

Text Box

Additional, Putative Mechanisms for Adaptive Fixation of Environmentally Induced mtDNA Modifications

To complete our understanding of how a changing environment might contribute to mtDNA evolution, we should consider the processes that can potentially lead to fixation not only of environmentally induced mutations (generated for example by boosted mtDNA replication or by environmental agents such as xenobiotics), but also of epigenetic modifications. With "environmentally induced mutations" we indicate mutations that are not adaptive characters per se, and, thus, they are not intended as "directed mutation" (see Charlesworth et al. 2017). Environmental induction of phenotypes (phenotypic plasticity) might represent a way for new traits to arise. In this light, environmentally induced mtDNA mutations and epigenetic modifications can be screened by natural selection and eventually be removed from or become fixed in a population, as in the case of the nuclear counterpart (Ehrenreich and Pfennig 2016). In this way, an environmentally induced phenotype has the potential to become an adaptive phenotype through a series of quantitative genetic changes (West-Eberhard 2003) that can be selected if the trait is favorable in that environment. This process is known as genetic accommodation (Moczek 2007; Moczek et al. 2011) and in its extreme form it is defined as genetic assimilation, that is, when plasticity decreases up to trait fixation (the trait becomes expressed constitutively in a population) (Waddington, 1952, 1953; Pigliucci et al. 2006; Ehrenreich and Pfennig 2016; Nishikawa and Kinjo 2018).

Although there is no evidence so far that these processes are relevant to mitochondrial genome evolution, they might represent putative mechanisms fixing mitochondrial genetic and epigenetic modifications. In this way, also regulatory components of mitonuclear interactions, like epigenetic modifications that confer some advantage in the new environment that caused them, represent targets on which genetic accommodation could act (e.g., Jones and Robinson 2018). Indeed, cases in which genes appear to be "followers" in the origins of novel traits were considered convincing, not only in animals but also in plants (Schwander and Leimar, 2011; Schlichting and Wund 2014; Ehrenreich and Pfennig 2016).

Experimental evolution studies provide definitive evidence for demonstrating genetic accommodation occurrence. However, ascertaining whether or not genetic assimilation has contributed to character evolution in natural

populations has long been questioned (Simpson 1953; Wray et al. 2014; Ehrenreich and Pfennig 2016, and references therein): once a novel trait has evolved, it is not straightforward to establish whether genetic assimilation has driven such novelty (Hall, 1999). It was suggested to approach such problem by studying ancestral lineages to the lineage possessing the considered novel character (Ehrenreich and Pfennig 2016, and references therein): if the ancestral species expresses the trait only through plasticity, whereas it is expressed independently from the environment in the derived lineage, we can suggest that genetic assimilation occurred (for a list of studies that provide evidence for genetic accommodation, see Schlichting and Wund 2014). We found no example about genetic assimilation studies of mitochondrial modification, but we think dedicated studies will follow in the near future.

Conclusions

The literature reviewed here illustrates the great complexity of mitochondrial evolutionary biology. Recent studies are revealing that epigenetic marks that increase fitness can rise in frequency in a population, and these changes may result in novel morphology, behavior, or physiology, and ultimately reproductive isolation (e.g., Smith et al. 2016). At the mitochondrial level, it is clear that more research is needed to better understand the potential importance of mtDNA methylation and noncoding RNAs in the capacity of animals to adjust their phenotype to variations in the environment. In addition, the literature reviewed in this paper illustrates that animal mtDNAs, including the human mtDNA, have a larger functional repertoire than previously believed (Breton et al. 2014; Capt et al. 2016; Angers et al. 2019; Miller et al. 2020; Mortz et al. 2020). For example, a deeper examination of the human mitochondrial genome revealed the existence of more than 200 open reading frames of 20 amino acids (Angers et al. 2019). The functional potential is therefore enormous, even for such a small genome. It is highly probable that several other mitochondrial ncRNAs and micropeptides will be discovered and their study will certainly allow us to understand the fundamental mechanisms regulating mitochondrial transcription and translation, as well as further reveal the intimate metabolic link between the mitochondria and the cell. A whole new class of mitochondrial ncRNAs and proteins would also transform the way we study the molecular mechanisms leading to the development of diseases related to mitochondrial dysfunction. In addition, this would offer new therapeutic avenues for many pathologies. The study of small mitochondrial proteins will also facilitate our understanding of the process of origin of new genes, which are thought to contribute to evolutionary innovations (Samandi et al. 2017). In other words, species- or lineage-specific mitochondrial micropeptides and ORFan genes probably hold the key to many recent adaptations, but they remain, for the most part, still uncharacterized.

The sources of mtDNA mutations in animals are still a matter of debate but recent findings suggest that a predominant source comes from imperfect replicative fidelity and repair mechanisms (Melvin and Ballard 2017). The rate and pattern of mutation accumulation in the mtDNA vary greatly within and among animal groups and are influenced by processes at different levels (molecular, cellular, population levels) (see also text box). All these aspects need to be comprehended to interpret evolution and disease linked to the mtDNA. The complexity of mitochondrial biology makes it difficult to clearly assess and explain the mechanisms of mtDNA adaptive evolution (Ghiselli and Milani 2020), and consequently to predict the effects of environmental changes and the long-time responses of the organisms. Given the central role of mitochondria in morphologically complex (eukaryotic) organisms, it will be important to pursue the ambitious goal of elucidating mechanisms patterns of evolution to better understand adaptation in the light of a rapidly changing environment (see, e.g., Blier et al. 2014). Undoubtedly, the best approach is to integrate genetics/genomics, biochemistry, and physiology to explore the widest biodiversity possible (Ballard and Pichaud 2014; Havird, Weaver, et al. 2019; Milani and Ghiselli 2020).

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Data Availability

No new data were generated or analysed in support of this research.

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