Linking the mitochondrial genotype to phenotype: a complex endeavour

PHILOSOPHICAL TRANSACTIONS
OF THE ROYAL SOCIETY B
BIOLOGICAL SCIENCES

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Journal:	*Philosophical Transactions B*
Manuscript ID	RSTB-2019-0169
Article Type:	Introduction
Date Submitted by the Author:	16-Oct-2019
Complete List of Authors:	Ghiselli, Fabrizio; Università di Bologna, Dipartimento di Scienze Biologiche, Geologiche ed Ambientali Milani, Liliana; University of Bologna, Department of Biological, Geological and Environmental Sciences (BiGeA)
Issue Code (this should have already been entered but please contact the Editorial Office if it is not present):	LINKING
Subject:	Evolution < BIOLOGY, Genetics < BIOLOGY, Molecular Biology < BIOLOGY, Synthetic Biology < BIOLOGY, Cellular Biology < BIOLOGY, Biochemistry < BIOLOGY
Keywords:	mtDNA genetic variation, heteroplasmy, mitochondrial bottleneck, mitonuclear interactions, mtDNA editing, mitochondrial expression manipulation

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Statement (if applicable):
CUST_STATE_CONFLICT :No data available.

Authors' contributions

This paper has multiple authors and our individual contributions were as below

Statement (if applicable):
FG and LM contributed equally to this work.

Title Page

Linking the mitochondrial genotype to phenotype: a complex endeavour

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Abstract

Finding causal links between genotype and phenotype is a major issue in biology, even more in mitochondrial biology. First of all, mitochondria form complex networks, undergoing fission and fusion and we do not know how such dynamics influence the distribution of mtDNA variants across the mitochondrial network and how they affect the phenotype. Second, the non-mendelian inheritance of mitochondrial genes can have sex-specific effects and the mechanism of mitochondrial inheritance is still poorly understood so it is not clear how selection and/or drift act on mtDNA genetic variation each generation. Third, we still do not know how mtDNA expression is regulated; there is growing evidence for a convoluted mechanism which includes RNA editing, mRNA stability/turnover, post-transcriptional and post-translational modifications. Fourth, mitochondrial activity differ across species as a result of several interacting processes such as drift, adaptation, genotype-by-environment interactions, mitonuclear coevolution, epistasis. This issue will cover several aspects of mitochondrial biology along the path from genotype to phenotype, and it is subdivided into four sections focusing on

mitochondrial genetic variation, on the relationship among mitochondria, germ line, and sex, on the role of mitochondria in adaptation and phenotypic plasticity, and on some future perspectives in mitochondrial research.

Keywords: mtDNA genetic variation; heteroplasmy; mitochondrial bottleneck; mitonuclear interactions; mtDNA editing; mitochondrial expression manipulation.

Mitochondria: powerhouse and beyond

Eukaryotic life is powered by mitochondria, cytoplasmic organelles that originated through a unique endosymbiotic event that changed the history of life on Earth fostering the evolution of multicellular organisms (Lane and Martin, 2010; Martin et al., 2015). Once a proteobacterium, the mitochondrion retained the bulk of its original biochemical machinery, but its genome (mtDNA) underwent a massive reduction in size, and genes of the ancestral organelle relocated to the nucleus ("endosymbiotic gene transfer", Timmis et al., 2004). However, not all the genes moved to the nucleus: of the 1,000+ protein-coding genes estimated to have been present in the bacterial ancestor of mitochondria, all known extant mtDNAs retain a subset of 69 ancestral protein-coding genes (Sloan et al., 2018); animal mtDNAs typically encompass ~13 of such genes (but see Breton et al., 2014). Interestingly, the organelles performing oxidative phosphorylation (OXPHOS) retain a genome, and there is a remarkable conservation of genes encoding key OXPHOS subunits in mtDNAs across eukaryotes (Sloan et al., 2018). John F. Allen (1993) postulated that organelle genomes encoding core OXPHOS subunits are necessary for maintaining redox poise (Co-location for Redox Regulation, CoRR; Allen, 2015); under this light, the interaction of two or three genomes—or even more in some protists (Archibald, 2015)—is unavoidable in eukaryotic cells (Lane et al., 2013). The five multi-protein complexes responsible for OXPHOS are composed of subunits encoded by both nuclear and mitochondrial genomes, which need to coevolve despite their markedly different genetic features (Lane, 2011; Wolff et al., 2014). Because of such differences, and because of some peculiar characteristics of mitochondria, it is quite challenging to reconstruct the evolutionary dynamics and predict the outcomes of mitonuclear

interactions and coevolution, and it is particularly difficult to find causal links between genotype and phenotype in mitochondrial biology (Aanen et al., 2014; Ballard and Melvin, 2010; Dowling, 2014). First of all, differently from the nuclear genome, mtDNA is subject to non-mendelian (uniparental) inheritance, and the underlying mechanism is still poorly understood, namely it is not clear how drift and selection act on mtDNA genetic variation each generation (see Milani and Ghiselli, 2015). Second—albeit with large variation (Allio et al., 2017)—metazoan mtDNA experience a higher mutation rate which results in different evolutionary rates between the two genomes. Third, because of the high mtDNA copy number per cell/organelle, mutations result in a phenotypic effect only when exceeding a threshold level (usually >60% mutant vs wild-type), meaning that alleles can fluctuate at mid-low frequency in the mitochondrial gene pool without being "visible" to selection (Busch et al., 2014; Dowling, 2014; "buffering", or "threshold effect": Ghiselli et al., 2013; Milani and Ghiselli, 2015). Fourth, individual mitochondria do not exist as permanently distinct entities, but undergo rapid fission and fusion processes, exchanging proteins, mtDNA, and lipids. Fission produces new mitochondria and plays an important role in quality control and selective elimination of damaged organelles. The fission process yields functionally divergent mitochondria—with different membrane potential ($\Delta\psi m$)—and depolarized mitochondria are selectively degraded by mitophagy. On the other hand, fusion produces a network whose components share matrix content and electrochemical gradient, and it has been suggested as a mechanism by which mitochondria complement damaged organelles and compensate metabolic deficiencies (Busch et al., 2014; Chan, 2006; Kim et al., 2007; Twig et al., 2008a, 2008b; Youle and van der Bliek, 2012). The mtDNA is organized in nucleoids, discrete DNA-protein complexes which are present in multiple copies (hundreds to thousands) per cell. Nucleoids can be segregated across individual organelles in a cell, but, given the dynamic nature of the mitochondrial network, the association between nucleoids and their products, among nucleoids, and among products is temporary. Thus, it is of particular interest the situation—much more common than once believed (Dowling, 2014)—where different mtDNA variants are present in the same individual, a condition called heteroplasmy. For reasons that are still unknown, heteroplasmy seems to be unfavourable (but see Ghiselli et al., 2019; Lane, 2012), and it has been related to

physiological, cognitive, and behavioural complications in mice (Sharpley et al., 2012), and to human neurodegenerative diseases and common age-related disorders (Stewart and Chinnery, 2015). For the above-mentioned reasons, it is quite difficult to assess the distribution of mtDNA variants across the mitochondrial network, and how phenotype is affected. Indeed, the link between genotype (mtDNA) and phenotype (e.g.: OXPHOS activity) depends on the mobility of mtDNA and the diffusion of its products, so such link can show various degrees of 'leakyness' (Busch et al., 2014; Kowald and Kirkwood, 2011). Understanding the effects of heteroplasmy—and of mitochondrial genetic variability in general—is therefore a complex endeavour.

From genotype to phenotype

The life science community is getting increasingly aware of the great complexity of mitochondrial biology and evolution, a complexity that has been underestimated for a long time. Recently, mitochondrial biology is getting more attention from scientists across a wide range of disciplines, both basic and applied. Even the mass media have been engaged in mitochondria-related discussion, especially regarding the issue of "three-parent babies" (mitochondrial replacement therapy for *in vitro* fertilization). On the biomedical side, the central role of mitochondria in a substantial number of cellular processes implies that mitochondrial malfunctions cause a wide typology of diseases. Once considered rare, mitochondrial disease is now thought to affect 1 in 5,000 people, making it the second most commonly diagnosed, serious genetic disease after cystic fibrosis (source: Global Mitochondrial Disease Awareness Week website, http://gmdaw.org/). Linking the mitochondrial genotype with disease, predicting its presence, severity, heritability, and finding a therapy is quite a challenging endeavour.

We think this Issue represents a relevant contribution for multiple fields of study. The link between some of the basic research here reported and future applications might seem far-fetched. We disagree. We are convinced that life sciences reached a turning point, where new technologies and methods allow to study a wider range of organisms and to

compare the basis of their biological features. Comparative analyses across increasingly large samples of biodiversity, are the most powerful approach to understand the evolution and functioning of organisms. The models and approaches described in this Issue will contribute to highlight similarities and differences between known aspects of mitochondrial biology and features of new uprising models that will surely contribute to the overall picture. Indeed, we want to highlight the importance of using comparative methods in a wide range of organisms, and the new models here presented show features that can help understanding some obscure areas of mitochondrial biology (see: Milani and Ghiselli, this Issue; Havird et al., 2019).

The contributions included are the result of nearly three years of interactions and discussions among scientists working in the field of mitochondrial evolutionary biology. Most of the interactions happened during international meetings, the last being "Linking the mitochondrial genotype to phenotype: a complex endeavour" SMBE 2018 Yokohama, Japan. The purpose of this collaborative effort is to provide new perspectives and angles in the field of mitochondrial biology and evolution. Hopefully, the work of the group of scientists participating in this Issue will increase the future contributions from different disciplines of life sciences, encouraging new collaborations and generating discussions.

The Issue will cover several aspects of mitochondrial biology along the path from genotype to phenotype, with a special attention to non-model species. The contributions (see Table 1) are subdivided into four sections: 1) mitochondrial genetic variation; 2) the relationship among mitochondria, germ line, and sex; 3) the role of mitochondria in adaptation and phenotypic plasticity; and 4) some future perspectives on mitochondrial research.

1) MITOCHONDRIAL GENETIC VARIATION

Genetic variation is the engine of evolution, and this section highlights some focal points about how mitochondrial genetic variation arises and changes within individuals and across generations.

Schaack et al. (this Issue) investigate the challenges in estimating mutation rates given unknowns such as mtDNA effective population size and fixation probability of heteroplasmic mutations. A critical parameter in understanding rates of change is estimating the mitochondrial mutation rate (mtDNA MR). Despite its importance, this kind of estimate is overlooked. Mutation accumulation experiments are demanding and do not help in distinguishing the role of mutation from other evolutionary forces. mtDNA MRs depend on the rate of replication errors and unrepaired DNA damage, but since there are multiple copies of the mtDNA genome per mitochondrion and many organelles per cell, the fate of a given mutation also depends on its selective coefficient and selection effectiveness, relative to the likelihood of loss/fixation by genetic drift. Schaack et al. (this Issue) review the unique features of the mitochondrial genome that pose a challenge for accurate mutation rate estimation and discuss ways to overcome such challenges and understand mtDNA MRs variation within and between individuals, populations, and species. They underline that to understand how mtDNA MRs evolve it is essential to extend the analysis to non-model organisms and multiple genotypes per taxon.

Dubie et al. (this Issue) use *Caenorhabditis elegans* data to discuss proliferation and persistence of spontaneous selfish mitochondrial deletions. Mitochondrial genomes can sustain mutations that are detrimental to individual fitness but that proliferate because of a replicative advantage (hence “selfish”). Dubie et al. (this Issue) analyzed the fitness effects and population dynamics of a mitochondrial genome containing a novel 499-bp deletion in the *ctb-1* gene (Δctb-1). Δctb-1 reached a high heteroplasmic frequency, imposing a significant fitness cost compared to individuals bearing wild-type mitochondria. Deletion-bearing worms were rapidly purged within a few generations when competed against wild-type mtDNA bearing worms in experimental populations. In contrast, the Δctb-1 mitotype was able to persist in large populations comprising heteroplasmic individuals only. The data obtained within experimental lines subjected to severe population bottlenecks indicate a selfish drive. Indeed, levels of mitochondrial heteroplasmy are the product of mutation and selection at different levels of organization, and the use of single individual bottlenecks can eliminate the selection between individuals, revealing the contributions of selection and drift within the germline.

Heteroplasmy is the presence of different mtDNA variants within the same individual. The dynamics of heteroplasmic allele frequency among tissues of the human body is not well understood. Barrett et al. (this Issue) present data supporting a pronounced bottleneck in the mtDNA of human hair. By measuring allele frequency at heteroplasmic sites, Barrett et al. (this Issue) observed high variance in allele frequency among separate hairs from the same individual. These findings have important implications for understanding mtDNA variation across different tissues in the human body occurring during embryonic development and throughout the lifetime. The described population genetic modeling estimated the somatic bottleneck during embryonic follicle development of separate hairs to be much more drastic than somatic bottlenecks for blood and buccal tissues but comparable to the germline bottleneck, and that hair undergoes additional genetic drift before and after the divergence of mtDNA lineages of individual hair follicles. These findings have important implications for our understanding of mtDNA dynamics and also for forensics: heteroplasmic frequency may vary between hairs, and heteroplasmy may be present in the hair sample but absent in another tissue of the same individual.

2) MITOCHONDRIA, GERM LINE, AND SEX

Which mitochondria are inherited from one generation to the subsequent through the germline? Is it a random subset or a selected one? If selection occurs, when and how is it achieved? Strictly maternal inheritance (SMI) entails an asymmetry in the transmission mechanism between sexes: what are the consequences on mitochondrial evolution and on the two sexes?

Knorre (this Issue) reviews the role of mitochondrial dynamics in mtDNA quality control and proposes cases in which mtDNA can evade it. Mitochondria can show different $\Delta\psi_m$ on which mitochondrial quality-control mechanisms rely, distinguishing between functional and damaged mitochondria. Nonetheless, mutations that increase $\Delta\psi_m$ can evade such control even being deleterious. Knorre (this Issue) reviews recent findings on intracellular mtDNA quality control by mitophagy and discuss other mechanisms by which the nuclear genome can affect the competition of mtDNA variants in the cell, thus affecting heteroplasmy levels. He also examines the hypothesis that the zygote is the stage at

which mtDNA quality control takes place at the intracellular level. Mitochondrial dynamics are required to fulfil multiple functions, but these dynamics can disrupt the genotype-to-phenotype linkage at the intracellular level, thus preventing intracellular quality control of mtDNAs. Knorre (this Issue) suggests that this trade-off has been resolved by the evolution of a restriction of intracellular quality control to the germline.

Because of SMI, some Authors predict that mitochondrial quality control is less effective in males ("Mother's curse hypothesis", see Frank and Hurst, 1996; Gemmell et al., 2004). Bettinazzi et al. (this Issue) use the only known evolutionarily stable exception to SMI to investigate the link between mtDNA variants and sperm performance. Because of the strict maternal inheritance of mitochondria in animals, haplotypes that negatively affect male fertility can become fixed in populations. Doubly uniparental inheritance (DUI) of mitochondria is a stable exception, found so far in 100+ bivalve species which show two mtDNA lineages that evolve independently, transmitted separately, one by oocytes and the other by spermatozoa. Since the two DUI mitochondrial lineages are likely subject to different sex-specific selective pressures, the DUI system is a unique model to evaluate selection on sperm mitochondria for male functions, potentially contributing to male reproductive fitness. This study highlighted a significant divergence in sperm performance and partially in energy metabolism between DUI and SMI species. As sperm mitochondria in DUI species are not an evolutionary dead-end, male-specific energetic adaptations could reflect selection for both fertilization success and male mitotype preservation.

Nagarajan-Radha et al. (this Issue) present new data about how mitochondrial genetic variation exerts sex-specific effects on physiological function. According to the mother's curse hypothesis, maternal inheritance of mitochondria will facilitate the accumulation of mtDNA mutations that are harmful to males but benign/beneficial to females. These male-harming mutations are expected to differ across a population and to cause larger genetic variation and possibly larger phenotypic effects in males and/or having sexually antagonistic effects. Nagarajan-Radha et al. (this Issue) explore signatures of male-bias or sexual antagonism in the metabolic rate by measuring the effects of different mitochondrial haplotypes on the production of carbon dioxide across strains of *Drosophila melanogaster*, controlling for mass and activity. The study reports sex-specific (male-biased) effects of mtDNA haplotypes on metabolic rate, and a negative intersexual

correlation for metabolic rate across haplotypes consistent with the prediction that SMI enabled the accumulation of mutations that increase female fitness, but at the expense of male fitness. The Authors highlight the importance to address future research to a broader range of nuclear genetic and environmental contexts and also to other metazoan species.

3) MITOCHONDRIA, ADAPTATION, AND PHENOTYPIC PLASTICITY

Mitochondria have a central role in many fundamental processes of eukaryotic life, well beyond energy production, so it should not be surprising that they have been suggested to be involved in adaptive processes (James et al., 2016; Meiklejohn et al., 2007). However, the mechanisms underlying mitochondrial-driven adaptation are complex and subject of debate. This section deals with the contribution of mitochondria and mitonuclear interactions to adaptation, phenotypic plasticity, and complex phenotypes.

Rand and Mossman (this Issue) discuss how mitonuclear conflict and cooperation govern the integration of genotypes, phenotypes, and environments. The interaction between the mitochondrial and nuclear genomes under changing environments have pervasively influenced organism evolution. Indeed, mitochondria play crucial roles in signaling, altering how nuclear genes are expressed as phenotypes. These interactions are examples of genotype-by-environment (GxE) and gene-by-gene (GxG) interactions, producing context-dependent effects on the link between genotype and phenotype. Mitonuclear interactions have pleiotropic effects across numerous phenotypes and evidence from *Drosophila* and other organisms shows that mitonuclear interactions are common features of GxE and GxG. Rand and Mossman (this Issue) outline approaches that could help depicting the phenotypic and fitness landscapes in a nuclear-mitochondrial co-evolved unit and their relation to genetic variation. For example, the population-structure-mitonuclear-coadaptation hypothesis possibly explains why the breakdown of mitonuclear coadaptation is so evident in *Tigriopus*—highly structured inbred populations—but not in large outbred species such as *Drosophila*. The Authors underline

how mitonuclear interactions are important to understand the context-dependent effects underlying the architecture of complex phenotypes.

Camus et al. (this Issue) analysed the impact of mitonuclear interactions on life-history responses to diet in *D. melanogaster*. Since mitochondria influence resource allocation, severe incompatibilities between mitochondrial and nuclear genomes can have pervasive effects on both fitness and longevity. How milder deficits in mitochondrial function affect life-history trade-offs is less well understood. Camus et al. (this Issue) found that in closely related fly populations (in which the genetic distance in mtDNA is similar to human populations) mitonuclear interactions do have significant impact on life-history trade-offs, but these effects are not predictable by relatedness and depend on the nuclear background. Camus et al. (this Issue) analysed how mitonuclear interactions affect the trade-off between fecundity and longevity considering different mitochondrial DNA haplotypes against two contrasting nuclear backgrounds in response to different diets. Mitonuclear interactions had substantial effects on resource allocation and life-history trade-offs in *D. melanogaster* but did not reflect genetic distance between mitochondrial haplotypes, so their effects are inconsistent, thus not predictable by relatedness. These effects can vary greatly, such as between the two nuclear genotypes used in this study, thus the Authors define as hazardous to generalise from mtDNA interactions with a single nuclear background.

Havird et al. (this Issue) examined mitochondrial function during thermal acclimation in mayfly larvae (*Baetis* and *Drunella* spp.). Modifications in mitochondrial or nuclear-encoded genes can modulate mitochondrial function and underlie environmental adaptation. Environmentally-induced plasticity in mitochondrial function is also common, especially in response to thermal acclimation in aquatic systems. Havird et al. (this Issue) examined mitochondrial activity in mayfly larvae from high and low elevation mountain streams during thermal acclimation to ecologically relevant temperatures. They evaluated different respiratory states in isolated mitochondria, and cytochrome oxidase and citrate synthase activities. The data obtained suggest that montane insects may be more vulnerable to rapid climate change. Indeed, mitochondria from samples collected at a low elevation site, with highly variable temperatures, showed greater thermal tolerance than samples from a high elevation site with comparatively stable temperatures, according to

predictions of the climate variability hypothesis. The Authors discuss how mitochondrial phenotypes are more resilient than whole-organism phenotypes in the face of thermal stress and underline the complex relationships between mitochondrial and organismal genotypes, phenotypes, and environmental adaptation.

Elbassiouny et al. (this Issue) discuss adaptations for elevated metabolic burden in electric fishes. Indeed, the interest in understanding molecular adaptations that enable electric fishes to generate and detect electric fields relies also on the extent of energetic burden required that account for up to 20% of fish daily energy expenditure. Elbassiouny et al. (this Issue) investigated the molecular evolution of the OXPHOS complexes in the two most diverse clades of weakly electric fishes—South American Gymnotiformes and African Mormyroidea—using codon-based likelihood approaches. From the data obtained, they suggest that the usual strong constraint on mitochondrial OXPHOS variation is significantly reduced in electric compared to non-electric fishes, particularly for some OXPHOS complexes. The results presented are consistent with positive selection on the two fish branches associated with the independent evolutionary origins of electrogenesis, so the Authors suggest that adaptive evolution in the OXPHOS machinery may be associated with the evolution of bioelectrogenesis. This evidence is added to other evidence consistent with positive selection associated with major changes in physiology or ecology, such as that at the origins of bats and the evolution of powered flight: these studies highlight the utility of comparative analyses to reveal the molecular basis of adaptations that appear to be important in the evolution of novel sensory systems.

Mackenzie and Kundariya (this Issue) review plant adaptation and phenotypic plasticity involving organelle-mediated epigenetic reprogramming. Plants can disperse their progeny to different environments and they can incorporate epigenetics and transgenerational stability thus allowing a high level of resilience. These genetic network and chromatin features increase acclimation opportunity and allow these sessile organisms to survive environmental change. Interestingly, some of such adaptational versatility of plants arises from neofunctionalization of organelles and organellar proteins. Mackenzie and Kundariya (this Issue) describe plastid specialization and multi-functional organellar protein features that support and enhance plant phenotypic plasticity. Spatio-temporal regulation of plastid composition, unusual inter-organellar protein targeting and

retrograde signalling can facilitate multi-functionalization of existing proteins. The process of neofunctionalization of sequences transferred from organelles to the nucleus is discussed, since the evolution of mitochondria and plastids as highly specialized cellular compartments has increased the functional versatility of many nuclear-encoded organellar proteins by virtue of their dual targeting: the redirection of a protein to a new cellular location can indeed influence protein neofunctionalization. The Authors also refer to the cytoplasmic male sterility (CMS), a mechanism described in over 80 plant species, associated with the expression of novel mitochondrial genes arising from intragenic recombination.

4) FUTURE PERSPECTIVES

This section focuses on new and old challenges in mitochondrial biology, reviewing up-and-coming technologies that will improve our ability to study the link between genotype and phenotype by, for example, mtDNA editing and manipulation of mitochondrial gene expression. Finally, we point out the importance of investigating a wider range of biodiversity by enhancing basic, “curiosity driven” research, and applying the comparative approach.

Klucnika and Ma (this Issue) discuss the challenge of mapping and editing animal mitochondrial genomes. Sequence variation among mtDNA haplotypes influences traits as health and longevity, but also incurable mitochondrial diseases, ageing, and cancer. However, significant challenges hamper our ability to precisely map mtDNA variants responsible for traits, and to genetically modify mtDNA. Klucnika and Ma (this Issue) review the efforts in developing systems to map and edit mtDNA, such as how to induce/increase the basal recombination frequency, and how to use mito-nucleases to cut endogenous genome and cause their subsequent degradation. The Authors also discuss the use of *in vitro* modified mtDNA directly delivered for transformation—but no mtDNA transformation metazoan system has been established so far—and the use of cell models for creating mutants that would otherwise be homoplasmic lethal at the organismal or tissue level. The impossibility to reliably deliver nucleic acids into animal mitochondria is a huge barrier, preventing us, for example, from importing also RNA for

CRISPR-mediated mtDNA editing: the establishment of mitochondria-adapted CRISPR-Cas9 platform could prompt a revolution in mitochondrial genome engineering and our biological understanding of mitochondria and mtDNA.

Wallis et al. (this Issue) review new methods for studying the genotype-phenotype link by manipulating mitochondrial gene expression with engineered proteins. Many genome engineering tools used for nuclear genome modification cannot be used to study mitochondrial genetics due to the unusual structure and physiology of the mitochondrial genome. Although challenges in the manipulation of mitochondria persist, new approaches are developed to modify the levels of mutant mammalian mitochondrial DNA and mitochondrial RNAs. Wallis et al. (this Issue) reviewed methods—such as mitoREs, mtZFNs, mitoTALENs, and RNA-binding proteins engineered to target specific mitochondrial RNA—that enable to manipulate mtDNA and modulate mitochondrial gene expression, to track and visualise mitochondrial processes, and whose application and study may provide highly specific and customisable genetic tools that could be applied in future therapeutics.

Milani and Ghiselli (this Issue) ponder the potentials of non-model systems in mitochondrial research, highlighting promising candidates. The concept of model organism is discussed starting from the words by August Krogh, 1929—*"For a large number of problems there will be some animal of choice or a few such animals on which it can be most conveniently studied".* Model organisms and inductive reasoning are irreplaceable, but we have to face with the problem of overgeneralisation. How can we infer general concepts? The role of model organisms in comparative biology is discussed in terms of model-organism-based approach vs comparative method. In doing this, some concepts from philosophy already used in scientific disciplines are utilized, such as nomothetics, ideographics, and an unusual concept of class. Several animals are rising as models in mitochondrial research: killifish (*Fundulus spp.* and *Nothobranchius furzeri*), deer mice (*Peromyscus spp.*), naked mole-rats (*Heterocephalus glaber*), bats of the genus *Myotis*, the bird *Eopsaltria australis*, the crustacean *Tigriopus californicus*, and bivalve molluscs, are currently used for answering specific biological questions such as the role of mitochondria in ageing and environmental adaptation, mitonuclear interactions and coevolution, genomic conflicts, mitochondrial heteroplasmy and inheritance.

Acknowledgements

We gratefully thank: all the Authors that submitted a contribution for this Issue; all the Reviewers involved; the Senior Commissioning Editor Helen Eaton for her amazing job in coordinating everything; the Editorial Board for the suggestions to the initial proposal; William F. Martin and John F. Allen for guidance. The involvement of Authors in this Issue was driven by personal interactions at several international conferences from 2016 to 2018. We apologize with the many outstanding Authors in the field of mitochondrial evolutionary biology that were not included.

Funding

This work was supported by the Italian Ministry of Education, University and Research (MIUR) SIR Programme grant No. RBSI14G0P5 funded to L.M., MIUR FIR2013 Programme grant No. RBFR13T97A and the Canziani bequest funded to F.G., and "Ricerca Fondamentale Orientata" (RFO) funding from the University of Bologna to L.M. and F.G.

Author Contributions

FG and LM contributed equally to this work.

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Table 1. List of the papers included in this Special Issue

Reference (this Issue)	Type	Organism(s)	Scale(s)	Evolutionary topic	Finding
MITOCHONDRIAL GENETIC VARIATION					
Schaack et al.	Opinion piece	Eukaryotes	Organismal Cellular	Mitochondrial mutation rate (mtDNA MR)	Ways to overcome the challenges of mtDNA MR estimation, disentangling the role of mutation from other evolutionary forces acting within the cell, and its variation within and between individuals, populations, and species
Dubie et al.	Primary research	*Caenorhabditis elegans*	Population Organismal Molecular	Selfish mitochondrial genome	Population dynamics of selfish mtDNA are strongly influenced by the population size
Barret et al.	Primary research	*Homo sapiens*	Population Organismal Tissue	Heteroplasmy	High variance in heteroplasmic allele frequency among hairs from the same individual and implications for forensics
MITOCHONDRIA, GERM LINE, AND SEX					
Knorre	Review	Animals Fungi	Cellular Organelle	Intracellular mitochondrial quality-control mechanisms	Germline cells are under severe pressure to eliminate deleterious mtDNA variants; also, the zygote appears to be another stage at which mtDNA quality control takes place at the intracellular level
Bettinazzi et al.	Primary research	Bivalve molluscs	Cellular	Sperm performance and bioenergetics	Possible link between male-energetic adaptation, fertilization success, and paternal mitochondria preservation in DUI species
Nagarajan-Radha et al.	Primary research	*Drosophila melanogaster*	Population Organismal	Mitochondrial genetic variation and physiological function	Empirical support that maternal mitochondrial inheritance has led to the accumulation of a sex-specific genetic load within the mitochondrial genome, affecting metabolic rate and the evolution of sex-differences
MITOCHONDRIA, ADAPTATION, AND PHENOTYPIC PLASTICITY					
Rand & Mossman	Opinion piece	*Drosophila melanogaster*	Population Organismal	Genotype-by-environment (GxE) and gene-by-gene (GxG) interactions	Mitonuclear interactions are common features of GxE and GxG interactions and are an important model to better understand the context-dependent effects on the link between genotype and phenotype

Camus et al.	Primary research	*Drosophila melanogaster*	Population Organismal	Fitness and longevity	Mitonuclear interactions can have significant impact on life-history trade-offs, but their effects are not predictable by relatedness
Havird et al.	Primary research	*Baetis tricaudatus* *Baetis bicaudatus* *Drunella coloradensis* (mayflies)	Organismal Cellular Molecular	Environmentally-induced plasticity in mitochondrial function	Mitochondria of cold-adapted insects were sensitive to even moderate increases in temperature; those living in thermally variable environments had greater thermal tolerance
Elbassiouny et al.	Primary research	Gymnotiformes Mormyroidea (electric fishes)	Molecular	Molecular evolution of OXPHOS complexes	Evidence for convergent patterns of molecular evolution of mitochondrial OXPHOS genes in two different groups of electrogenic fishes
Mackenzie & Kundariya	Review	Plants	Organismal Organelle	Phenotypic plasticity in plants	Some of the underlying versatility of plants to adapt to abiotic and biotic stress emerges from neofunctionalization of organelles and organellar proteins
FUTURE PERSPECTIVES					
Klucnika & Ma	Review	Animals	Organismal Molecular	Map and edit mtDNA	The efforts in developing systems to induce/increase the basal recombination frequency, and the use of mito-nucleases, could finally lead to the establishment of mitochondria-adapted DNA editing platform
Wallis et al.	Review	Mammals	Population Organismal Molecular	Tools for manipulating mitochondrial gene expression	mtDNA sequence-specific DNA-binding and RNA-binding proteins tethered to various effector domains, show great promise as highly specific, customisable genetic tools for mitochondrial research
Milani & Ghiselli	Opinion piece	Model/Non-model animals	Organismal	Perspective in mitochondrial research	The comparative method and new animal models have the potential to address still unanswered biological questions