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Publication Date

2018-12-26

DOI

10.1111/brv.12493

Peer reviewed

1 **Assessing the fitness consequences of mitonuclear interactions in**
2 **natural populations**

3

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19 **Short title: fitness consequences of mitonuclear interactions**

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22

23 ABSTRACT

24 Metazoans exist only with a continuous and rich supply of chemical energy from oxidative
25 phosphorylation in mitochondria. The oxidative phosphorylation machinery that mediates energy
26 conservation is encoded by both mitochondrial and nuclear genes, and hence the products of
27 these two genomes must interact closely to achieve coordinated function of core respiratory
28 processes. It follows that selection for efficient respiration will lead to selection for compatible
29 combinations of mitochondrial and nuclear genotypes, and this should facilitate coadaptation
30 between mitochondrial and nuclear genomes (mitonuclear coadaptation). Herein, we outline the
31 modes by which mitochondrial and nuclear genomes may coevolve within natural populations,
32 and we discuss the implications of mitonuclear coadaptation for diverse fields of study in the
33 biological sciences. We identify five themes in the study of mitonuclear interactions that provide
34 a roadmap for both ecological and biomedical studies seeking to measure the contribution of
35 intergenomic coadaptation to the evolution of natural populations. We also explore the wider
36 implications of the fitness consequences of mitonuclear interactions, focusing on central debates
37 within the fields of ecology and biomedicine.

38

39 *Key words:* mitochondria, coadaptation, coevolution, epistatic interactions, gene flow, speciation,
40 mitochondrial medicine, fitness.

41

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64 **I. INTRODUCTION**

65 Life depends on efficient production of useable energy. The substantial energy needs of most

66 metazoans are met by the oxidative phosphorylation (OXPHOS) system embedded within the

67 inner membrane of mitochondria, which produces approximately 90% of the ATP available to

68 cells (Lane & Martin, 2010). The enzyme complexes that mediate OXPHOS are comprised of

69 numerous polypeptide subunits. Most of these subunits are encoded by nuclear genes and are
70 transported into mitochondria. However, multiple proton-translocating subunits (13 in bilaterian
71 animals) are encoded by the mitochondrial DNA (mtDNA) (Bar-Yaacov, Blumberg & Mishmar,
72 2012). Consequently, energy production in eukaryotes must rely on a critical set of interactions
73 between genes that span two distinct genomes (Rand, Haney & Fry, 2004; Wolff *et al.*, 2014;
74 Hill, 2015).

75 Because the products of mitochondrial genes play a key role in enabling core respiratory
76 processes, it was long assumed that variants that appeared within the mtDNA sequence would be
77 quickly removed by purifying selection (Avisé, 2004). This assumption has been supported by
78 analyses of ratios of nonsynonymous (amino acid-changing) to synonymous (putatively silent)
79 mutations across key mitochondrial genes (mt genes) of metazoans (Rand, 2001; Stewart *et al.*,
80 2008a; Nabholz, Ellegren & Wolf, 2013; Popadin *et al.*, 2013; Zhang & Broughton, 2013).
81 Accordingly, a generation of evolutionary biologists, from the 1980s onwards, worked under the
82 purview that the mitochondrial sequence variation segregating within or among populations was
83 selectively neutral (Ballard & Whitlock, 2004; Dowling, Friberg & Lindell, 2008; Ballard &
84 Pichaud, 2014).

85 By the mid-1990s, however, several studies had emerged that refuted the strict neutrality
86 of variation in mtDNA sequence (Ballard & Kreitman, 1994; Nachman, Boyer & Aquadro, 1994;
87 Nachman *et al.*, 1996; Rand, Dorfsman & Kann, 1994; Pichaud *et al.*, 2012). In subsequent
88 years, a series of experimental studies highlighted numerous cases in which the genetic variation
89 found within the mitochondrial genome was clearly non-neutral (i.e. functional), with pervasive
90 effects on metabolic function (Willett, 2008; Arnqvist *et al.*, 2010; Pichaud *et al.*, 2012; Barreto
91 & Burton, 2013a; Bock, Andrew & Rieseberg, 2014; Wolff *et al.*, 2016) and the expression of

92 life-history traits (James & Ballard, 2003; Rand, Fry & Sheldahl, 2006; Clancy, 2008; Dowling
93 *et al.*, 2009; Dowling, Meerupati & Arnqvist, 2010; Ma *et al.*, 2016; Roux *et al.*, 2016).
94 Furthermore, these non-neutral mitochondrial effects often exhibited evidence of epistatic
95 interactions with nuclear genes (Dobler *et al.*, 2014; Wolff *et al.*, 2014), consistent with the
96 premise that interactions between mitochondrial and nuclear genomes drive the functionality of
97 OXPHOS.

98

99 **II. EVIDENCE FOR COEVOLUTION OF MITOCHONDRIAL AND NUCLEAR** 100 **GENOMES**

101 Research efforts have since aimed to dissect the evolutionary mechanisms that generate
102 functional mitochondrial variation, and much emphasis has been placed on the potential for
103 accumulation of mildly deleterious mutations in mtDNA (Lynch & Blanchard, 1998; Neiman &
104 Taylor, 2009). The notion of a high mitochondrial mutation load runs contrary to the expectation
105 that strong purifying selection would effectively prevent the accumulation of non-neutral
106 variants. However, studies in mutant mouse models have suggested that purifying selection may
107 only be fully effective at removing non-synonymous mtDNA mutations from the female germ
108 line when these mutations confer severely pathogenic effects (Fan *et al.*, 2008; Stewart *et al.*,
109 2008*b, a*). Mitochondrial mutations of moderate effect, including those in transfer RNA (tRNA)
110 and ribosomal RNA (rRNA) genes, have been reported to escape selection and be transmitted
111 across generations (Alston *et al.*, 2017; Barreto *et al.*, 2018). When combined with the
112 observation that mitochondrial genes of many eukaryotes mutate at much higher rates than
113 nuclear genes (Brown, George & Wilson, 1979; Lynch, 1997; Smith & Keeling, 2015; Havird &
114 Sloan, 2016), and that these mutations reside in a genome that has traditionally been thought to

115 experience very low rates of recombination (i.e. an efficient mechanism of preventing mutational
116 accumulation) (Hagström *et al.*, 2014), there would appear to be ample opportunity for mutations
117 to accumulate and contribute to functional mitochondrial variation.

118 If left unchecked, mutational erosion of the mitochondrial genome would quickly lead to
119 degradation of energy production and metabolic homeostasis (Lynch & Blanchard, 1998). It is
120 therefore theorized that mutational erosion should create selection for nuclear genotypes able to
121 offset the negative metabolic effects caused by mtDNA mutations (Rand *et al.*, 2004). Indeed,
122 several studies have now identified signatures of complementary changes in interacting nuclear-
123 encoded genes that have evolved in conjunction with sequence changes in the mitochondrial
124 genome (Osada & Akashi, 2012; Barreto & Burton, 2013*b*; Sloan *et al.*, 2014; Havird *et al.*,
125 2015*b*, 2017; Van Der Sluis *et al.*, 2015; Barreto *et al.*, 2018; Yan, Ye & Werren, 2018). These
126 tandem changes appear consistent with a model of compensatory mitonuclear coevolution. Under
127 this model, the mitochondrial genome would provide a mutational pressure that precipitates
128 coadaptation between mitochondrial and nuclear genomes within populations (Ellison & Burton,
129 2008*b*; Barreto & Burton, 2013*b*; Yee, Sutton, & Dowling, 2013; Havird *et al.*, 2015*b*).

130 The importance of compensatory coevolution between the nuclear and mitochondrial
131 genomes is, however, a topic of current debate (Sloan, Havird & Sharbrough, 2017), and the
132 argument that an asexual and uniparental mode of inheritance makes mtDNA prone to
133 deleterious mutation accumulation has been criticized on both empirical and theoretical grounds
134 (Popadin *et al.*, 2013; Zhang & Broughton, 2013; Cooper *et al.*, 2015; Christie & Beekman,
135 2016). Long-held views regarding the effective haploidy, low effective population size, and
136 inefficient selection of the mitochondrial genome are being challenged (Ballard & Whitlock,
137 2004; Cooper *et al.*, 2015). Indeed, the fact that mtDNA molecules exist in hundreds to

138 thousands of copies per cell suggest it might be better viewed as a polyploid genome (Greaves &
139 Taylor, 2006). Across eukaryotes, there are variable rates of biparental inheritance of mtDNA
140 and at least occasional recombination between divergent mtDNA molecules (Greiner, Sobanski
141 & Bock, 2015; Ma & O'Farrell, 2015). In bilaterian animals, there is also a genetic bottleneck in
142 mtDNA copy number through the germ line (Stewart & Larsson, 2014), which could provide an
143 effective means by which selection can effectively purge primordial germ cells carrying mtDNA
144 molecules with pathogenic mutations (Burr, Pezet & Chinnery, 2018). Together, these factors
145 (polyploidy, some degree of biparental inheritance, recombination, and a genetic bottleneck
146 during oogenesis) are providing new insights into the dynamics of selection that shape
147 trajectories of mitochondrial genome evolution.

148 There is also growing interest in the role of *adaptive* changes in mitochondrial genomes,
149 and recent research suggests that a substantial fraction of non-synonymous substitutions in
150 mitochondrial genes may be driven by positive selection (James, Piganeau & Eyre-Walker,
151 2016). Because of the intimate functional integration between nuclear and mitochondrial
152 genomes, adaptive changes in mtDNA are likely to have epistatic effects and shift selection
153 pressures on the nuclear genome. Indeed, many examples that have been interpreted as
154 supporting a model of compensatory mitonuclear coevolution (Osada & Akashi, 2012; Barreto &
155 Burton, 2013b; Sloan *et al.*, 2014; Havird *et al.*, 2015b, 2017; Van Der Sluis *et al.*, 2015; Yan *et*
156 *al.*, 2018) are also consistent with other forms of mitonuclear coevolution that do not depend on
157 the accumulation of deleterious mitochondrial mutations (Sloan *et al.*, 2017). Regardless of the
158 relative contributions of deleterious, neutral, and beneficial changes in triggering the
159 coevolutionary process, natural selection is expected to favour beneficial combinations of alleles

160 spanning mitochondrial and nuclear genomes and give rise to coadapted mitonuclear genotypes
161 (Rand *et al.*, 2004; Burton, Pereira & Barreto, 2013; Wolff *et al.*, 2014; Hill, 2015).

162 Herein, we emphasize that further research attention is required to decipher the biological
163 significance of mitonuclear interactions. Despite the ubiquity of co-functioning mitochondrial
164 and nuclear genes, our understanding of the contribution of mitonuclear genetics to metazoan
165 fitness remains incomplete, and most insights are from laboratory-based studies of model
166 organisms. A focus on mitonuclear coadaptation is likely to contribute tangibly to our
167 understanding of basic ecological concepts, such as speciation and the dynamics of sexual
168 conflict (Hill, 2015; Wolff *et al.*, 2016). Furthermore, the implications of such interactions might
169 resonate beyond the evolutionary and ecological sciences, into the realm of biomedicine
170 (Mishmar & Zhidkov, 2010; Wallace, 2010; Dowling, 2014; Gershoni *et al.*, 2014). To inform
171 future research directions, we identify and discuss five themes that have emerged from the study
172 of mitonuclear interactions over the past two decades (Table 1).

173

174 **II. IMPLICATIONS OF MITONUCLEAR INTERACTIONS SPANNING ECOLOGY** 175 **AND BIOMEDICINE**

176 It has been proposed that the necessity for mitonuclear coadaptation for cellular respiration may
177 underlie a range of core evolutionary innovations and concepts. These include the evolution of
178 sex and two sexes in eukaryotes (Hadjivasiliou *et al.*, 2013; Havird, Hall & Dowling, 2015a), the
179 evolution of a sequestered germ line in bilaterian animals (Radzvilavicius *et al.*, 2016), climate
180 and resource adaptation (Camus *et al.*, 2017; Sunnucks *et al.*, 2017), sexual selection (Hill &
181 Johnson, 2013; Hill, 2018), and speciation (Dowling *et al.*, 2008; Burton & Barreto, 2012; Hill,
182 2016).

183 The role of mitonuclear interactions in mediating the process of speciation is currently a
184 major area of scientific research and debate (Hill, 2017; Sloan *et al.*, 2017). It has been proposed
185 that independent coevolution of mt and nuclear genes in isolated populations could lead to
186 uniquely coadapted sets of genes that are not compatible with the coadapted mt and nuclear
187 genes of other populations. If this is the case, gene flow and hybridization events between
188 diverging populations could produce negative phenotypic outcomes due to Dobzhansky–Muller
189 incompatibilities underpinned by mitonuclear interactions (Levin, 2003; Dowling *et al.*, 2008;
190 Gershoni, Templeton & Mishmar, 2009; Burton & Barreto, 2012; Hill, 2017). In theory,
191 therefore, population divergence driven by mitonuclear interactions represents a plausible model
192 underlying the evolution of reproductive isolation between incipient populations, and ultimately
193 speciation. However, the hypothesis that mitonuclear coadaptation plays a direct and general role
194 in driving speciation processes remains controversial and requires further investigation (Gershoni
195 *et al.*, 2009; Chou & Leu, 2010; Burton & Barreto, 2012; Bar-Yaacov *et al.*, 2015; Eyre-Walker,
196 2017; Hill, 2017; Sloan *et al.*, 2017).

197 The fitness consequences of mitonuclear interactions are also relevant for human
198 medicine. Ongoing research is exploring the significance of diverse mitochondrial haplotypes
199 across human populations (Mishmar *et al.*, 2003; Wallace, 2010). An emerging research focus in
200 biomedicine considers whether human phenotypes are dependent on the nuclear background in
201 which mitochondrial haplotypes are expressed (Levin *et al.*, 2014). Notably, mitonuclear
202 interactions have also been implicated as putative contributors to health outcomes associated
203 with the emerging germline therapy of mitochondrial replacement (Reinhardt, Dowling &
204 Morrow, 2013; Morrow *et al.*, 2015; Dobler *et al.*, 2018) (Fig. 1). Mitochondrial replacement is a
205 modified form of *in vitro* fertilization that could enable prospective mothers that suffer from

206 mtDNA-induced mitochondrial diseases to produce offspring that are free from the mother's
207 mtDNA mutations (Tachibana *et al.*, 2009, 2013; Craven *et al.*, 2010). The technique pairs a
208 patient's nuclear chromosomes, or fertilized pronuclei, with a healthy complement of donor
209 mitochondrial genes inside the donor's oocyte. Concerns have been raised, however, that the
210 approach may create novel combinations of patient nuclear genotype and donor mtDNA
211 haplotype that have not been previously tested by natural selection and that may lead to
212 unanticipated negative outcomes (Morrow *et al.*, 2015; Dobler *et al.*, 2018). The potential
213 negative effects of producing novel mitonuclear combinations in human oocytes remain widely
214 debated (Reinhardt *et al.*, 2013; Chinnery *et al.*, 2014; Morrow *et al.*, 2015; Sloan, Fields &
215 Havird, 2015; Eyre-Walker, 2017; Rishishwar & Jordan, 2017; Zaidi & Makova, 2018), with a
216 recent meta-analysis presenting evidence that suggests mitonuclear interactions are likely to
217 affect health outcomes in humans, and indeed seem to be associated with stronger effect sizes in
218 humans than other metazoans (Dobler *et al.*, 2018).

219

220 **III. EMERGING THEMES IN STUDIES OF MITONUCLEAR COADAPTATION**

221 An improved understanding of mitonuclear evolutionary dynamics is required to elucidate
222 the role of mitonuclear interactions in ecological processes such as speciation and biomedical
223 procedures like mitochondrial replacement therapy. Below we detail five themes that we believe
224 should guide future research in this field. These themes have not been synthesized previously
225 into a single framework or readily acknowledged in the current literature. However, we believe
226 each of these themes deserves consideration, particularly when applied to inferences from natural
227 populations and to implications beyond the fields of ecology and evolution.

228

229 **(1) Theme 1: selection for mitonuclear compatibility should be strong and should exist**
230 **across all stages of ontogeny**

231 Selection for efficient mitochondrial function, and hence mitonuclear compatibility, is expected
232 to be intense, and it might well begin as early as oogenesis, with massive selection on cells in the
233 germ line (De Fanti *et al.*, 2017; Krakauer & Mira, 1999; Fan *et al.*, 2008; Stewart *et al.*, 2008a;
234 Dowling, 2014; Radzvilavicius *et al.*, 2016). When there are multiple mitochondrial genotypes
235 per cell, natural selection becomes inefficient in either eliminating deleterious genotypes or
236 promoting highly functional genotypes (Radzvilavicius *et al.*, 2016). In bilaterian animals, germ
237 line selection on mt genotypes is therefore seemingly facilitated by a well-documented
238 bottleneck in the number of mtDNA molecules per primary oocyte (Wai, Teoli & Shoubridge,
239 2008; Stewart & Larsson, 2014). This mitochondrial genetic bottleneck enables natural selection
240 to screen oocytes based on their metabolic integrity, underpinned by their mtDNA genotype
241 (which should be fairly homogenous due to the bottleneck) and modulated by an effect of the
242 diploid nuclear genomic background (Cree *et al.*, 2008; Wai *et al.*, 2008). During these stages,
243 there is also a massive cull in the population of oocytes *via* a process called atresia. Together,
244 this provides two levels by which selection might screen for best-functioning mitochondria.

245 Emerging evidence supports the contention that the mitochondria are active within primordial
246 germ cells and developing oocytes (Ge *et al.*, 2012; Kasashima, Nagao & Endo, 2014; Hayashi
247 *et al.*, 2017). Thus, we hypothesize that only those oocytes with full capacity for efficient
248 respiratory function, requiring compatible mitonuclear genotypes, reach maturity (Dumollard,
249 Duchen & Carroll, 2007; Stewart & Larsson, 2014). Germ line selection could well represent a
250 core mechanism favouring the transmission of compatible mitonuclear genotypes across
251 generations (Lane, 2005; Morrow *et al.*, 2015; Radzvilavicius *et al.*, 2016), preventing the inter-

252 generational mutational meltdown of the mitochondrial genome predicted by theory (Lynch *et*
253 *al.*, 1993; Lynch & Blanchard, 1998; Stewart *et al.*, 2008a; Cooper *et al.*, 2015), but more data
254 are needed to assess the importance of selection on germ lines. Furthermore, this process can be
255 completed within the developing female foetus during embryogenesis. In humans, this occurs
256 decades before the female is likely to mate and produce her own offspring.

257 In addition, there may be considerable opportunity for selection to act on mitonuclear
258 interactions during the development of cells from spermatogonia to mature sperm cells, but this
259 topic seems not to have been investigated. In humans, a single ejaculate contains approximately
260 10^8 sperm, with only one sperm fertilizing an egg. Again, the difference between a successful
261 and unsuccessful sperm is not random, and strong postcopulatory selection will act on the male
262 ejaculate (Simmons, 2001). Swimming speed and endurance, capacity to cope with chemical
263 barriers, and ability to penetrate the egg faster than competitors dictate success (Snook, 2005;
264 Pizzari, 2009), and sperm derive at least part of the energy that underlies these functions from
265 OXPHOS (Ruiz-Pesini *et al.*, 2007). With few exceptions (Barr, Neiman & Taylor, 2005), the
266 mitochondria that power sperm are not transmitted to offspring. By contrast, the paternal nuclear
267 genome, which includes over 1000 nuclear-encoded mitochondrial (N-mt) genes, is transmitted
268 (Calvo & Mootha, 2010). These N-mt genes create the majority of the mitochondrial proteome,
269 and many interact closely with the mitochondrial-encoded gene products, such that strong
270 selection on mitochondrial function will plausibly lead to strong selection on paternal N-mt
271 genotypes in the sperm that enable cofunction with the common mt genotype of that population.

272 Selection for mitonuclear compatibility should then continue at every developmental stage
273 following fertilization (Chan, 2006; Latorre-Pellicer *et al.*, 2016). For instance, in mammals, not
274 all zygotes will successfully implant in the uterine wall; many developing embryos are

275 spontaneously aborted; many individuals die during early development; and only a portion of the
276 offspring born into the population will survive to reproductive maturity and succeed in procuring
277 a mate and ultimately in producing viable offspring themselves. The chances of surviving
278 through all of these stages of selection are minimal. The haploid gamete that survives pre-zygotic
279 selection, and then as a diploid genome survives all post-zygotic phases of selection through its
280 way, can be considered a one-in-a-million winner in the lottery of life. We propose that at each
281 of these life stages, selection for mitonuclear compatibility could be key. That is, although
282 mitochondrial and nuclear alleles are unlinked and segregate randomly, strong selection through
283 ontogeny could ensure that each adult in the population harbours a fully compatible mitonuclear
284 genotype. The majority of the competition that underlies such selection is, however, difficult to
285 detect unless one makes extremely careful and detailed observations across life stages. Such
286 studies should be a priority for future research.

287

288 **(2) Theme 2: mitonuclear coadaptation is manifested in mitochondrial physiology**

289 Under a model of mitonuclear coadaptation, mismatching of coevolved mitonuclear genomes
290 should lead to reduced organismal fitness specifically due to compromised mitochondrial
291 function and disturbed bioenergetics (Gershoni *et al.*, 2009). However, given that validation can
292 be operationally very challenging, observed dysfunction in crosses between divergent
293 populations is seldom linked to specific mitonuclear incompatibilities and loss of mitochondrial
294 function. Most experimental designs aimed at disrupting coevolved mitonuclear genotypes, such
295 as crossing individuals from genetically divergent populations, also disrupt coevolved epistatic
296 combinations of nuclear genes, and in such studies nuclear–nuclear rather than mito–nuclear
297 gene interactions might have the largest effect on organismal fitness. While reciprocal crossing

298 designs go some way towards removing the confounding effects of nuclear–nuclear interactions,
299 by shifting the focus onto putative mitonuclear interactions, such designs come with a caveat in
300 species with genetic sex determination because the genotype of offspring of the heterogametic
301 sex will differ across each of the reciprocal crosses. Furthermore, offspring produced by these
302 crosses are prone to effects from other extranuclear sources of variance, such as differences in
303 the microbiome profiles of the mothers or, in some arthropods, cytoplasmic incompatibility
304 caused by *Wolbachia* infection (Werren, Baldo & Clark, 2008; Schaefer, Nadeau & Wray, 2015).

305 One method for isolating mitonuclear effects is to replace the mtDNA from one lineage with
306 the mtDNA from another lineage thereby creating novel mitonuclear gene combinations. Such
307 genomic rearrangement can be achieved either by backcrossing over multiple generations (taking
308 advantage of the maternal inheritance of mtDNA, but biparental inheritance of nuclear genes) or
309 by using genetic tools that suppress recombination to enable chromosome substitution across
310 generations (Dowling *et al.*, 2008). The outcome of such manipulations is the creation of a
311 genetic strain of organism that possesses a novel mitonuclear genotype, in an otherwise intact
312 diploid nuclear background. This combination of approaches allows the researcher to home in on
313 the role of mitonuclear interactions in maintaining organismal function. Such approaches are,
314 however, only possible in study species that are easily propagated in the laboratory environment
315 and that have short generations.

316 One key prediction that can be tested in natural populations is that mitonuclear
317 incompatibilities should have disproportionate effects on mitochondrial function. Moreover,
318 mitochondrial physiology should be affected in a predictable manner according to the specific
319 components that are influenced by incompatibilities (Burton *et al.*, 2013). A prime testing ground
320 for assessment of mitonuclear effects distinct from nuclear–nuclear effects is comparison

321 between OXPHOS complexes composed of both mt- and nuclear-encoded subunits, and
322 complexes with only nuclear-encoded subunits. In many eukaryotes, Complex II (succinate
323 dehydrogenase) is made up entirely of nuclear-encoded proteins, while other complexes
324 responsible for OXPHOS function are chimeric assemblies of nuclear- and mitochondrial-
325 encoded proteins (Rand *et al.*, 2004) (Fig. 2). Complex II function should therefore remain stable
326 (or may even increase as a compensatory measure) regardless of altered mitonuclear interactions,
327 while Complex I (Nicotinamide adenine dinucleotide-dehydrogenase) and Complex IV
328 (cytochrome *c* oxidase) activity are predicted to vary with the mitochondrial genomic
329 background. This prediction was supported in studies with copepods and fruit flies in which
330 mitochondrial and nuclear genotypes from divergent populations were introgressed. In the fruit
331 fly experiment, when hybrids were created that carried nuclear-encoded genes for a tRNA
332 synthetase from *Drosophila melanogaster* and mt-encoded tRNA from *D. simulans*, poor
333 cofunctioning of these non-coadapted mt and N-mt genes caused impairment of translation of
334 mt-encoded OXPHOS subunits with significant effects on Complexes I, III, and IV but no effects
335 on Complex II (Meiklejohn *et al.*, 2013). In the copepod experiment, hybrid crosses between
336 divergent populations of *Tigriopus californicus* reduced activities of OXPHOS Complexes I, III,
337 IV, and V but not Complex II (Ellison & Burton, 2006). These are among the clearest
338 demonstrations of hybrid dysfunction resulting from mito–nuclear interactions because they
339 compellingly indicate that breakdown is only manifested for enzymes under dual control of both
340 nuclear and mitochondrial genomes.

341 In addition to measuring activities of OXPHOS complexes, a range of other approaches can
342 provide insight into mitochondrial physiology. Notably, production of ATP and generation of
343 reactive oxygen species (ROS) have been shown to vary with mitochondrial genotype (Ellison &

344 Burton, 2006, 2008a; Estes *et al.*, 2011; Barreto & Burton, 2013a; Hicks, Denver & Estes, 2013;
345 Barreto, Pereira & Burton, 2015; Latorre-Pellicer *et al.*, 2016). In tractable systems, detailed
346 examination of respiration profiles from isolated mitochondria could also reveal the mechanistic
347 basis for lower fitness in compromised individuals (Chung, Bryant & Schulte, 2017; Mowry *et*
348 *al.*, 2017; Zhang *et al.*, 2018). While rarely adopted, whole-organism measurements of basal and
349 maximal metabolic rates may also be useful for assessing the fitness consequences of
350 mitonuclear interactions (Sunnucks *et al.*, 2017).

351

352 **(3) Theme 3: generational delays**

353 Research into the capacity for mitonuclear incompatibilities to evolve in natural populations
354 suggests that, when such incompatibilities emerge, they may not be revealed until F2 and
355 subsequent generations (Burton, Ellison & Harrison, 2006) (Fig. 3). The apparent lack of
356 mitonuclear incompatibilities in the F1 generation from some crosses may arise because
357 offspring receive a full haploid copy of each autosomal chromosome from each parent. Under
358 this model, a full haploid maternal complement of nuclear genes would often be sufficient to
359 maintain mitonuclear-mediated organismal function. As we outlined in Theme 1, a
360 reproductively mature female has survived multifaceted phases of selection, from both pre-
361 fertilization to post-fertilization and across the entire ontogeny. As a result of relentless selection
362 throughout ontogeny, a mother's nuclear genotype should be predicted to function well with her
363 mitochondrial genotype. Therefore, even if the father's nuclear genetic contribution to the
364 offspring genotype exhibits incompatibility with the maternal mitochondrial haplotype, effects of
365 mitonuclear incompatibility may not manifest in the F1 generation because the compatible
366 nuclear alleles provided by the female may mask the effects of less-functional variants provided

367 by the male (Burton & Barreto 2012; Stelkens, Schmid & Seehausen, 2015). If F1 hybrids are
368 crossed to create F2 hybrids, however, the segregation of diploid nuclear genes will generate
369 recombinant genotypes, with some individuals receiving two paternal copies at a given nuclear
370 locus.

371 The degree to which incompatibilities are masked in the F1 generation will depend on
372 dominance relationships among alleles (Turelli & Orr, 2000; Raj *et al.*, 2010) because it is
373 expected that both sets of alleles will be expressed in F1s. The degree of masking should also
374 depend on whether the relevant nuclear genes are autosomal or sex-linked (Hill & Johnson,
375 2013; Hill, 2014). Researchers also face a challenge in separating the effects of any deleterious
376 mitonuclear interactions from the potentially offsetting benefits of heterosis ('hybrid vigour')
377 that are often seen in F1s, and which is attributable to the masking of deleterious recessive
378 mutations within the nuclear genome (Edmands, 2007).

379 The best description of generational delays in the negative effects of mitonuclear
380 incompatibilities in hybrid crosses comes from studies of the copepod, *T. californicus*, which
381 have no sex chromosomes. When individuals from genetically divergent populations are crossed,
382 F1 offspring typically exhibit a fitness gain relative to the parental populations. In F2 and later-
383 generation recombinants, however, hybrids suffer a fitness cost. Furthermore, full fitness is
384 restored when F2 hybrid females are backcrossed to males from the maternal lineage (Ellison &
385 Burton, 2008b). The fitness advantage in the F1 generation in this example can be ascribed to
386 heterosis associated with the creation of offspring exhibiting genome-wide heterozygosity in the
387 diploid nuclear genome. There appear to be no negative effects of interpopulation hybridization
388 at this F1 stage. Yet, F2 offspring clearly have reduced fitness relative to parental populations,

389 and given that fitness outcomes are only restored upon backcrossing with the maternal
390 population, this implicates mitonuclear incompatibilities as drivers of these effects (Fig. 3).

391

392 **(4) Theme 4: mitonuclear incompatibilities can be created by single base substitutions and**
393 **are not limited to protein-encoding genes**

394 The overall genetic divergence of individuals can be a misleading index of mitonuclear
395 compatibility. Many of the changes that distinguish mitochondrial haplotypes are likely to be
396 neutral or nearly so. Only a subset of nuclear gene products are transported to the mitochondrion,
397 and only a small proportion of these nuclear genes whose products function in mitochondria will
398 interact closely with mitochondrial gene products (Burton & Barreto, 2012; Aledo *et al.*, 2014).
399 Thus, if mt and N-mt genes are under strong purifying selection, then there is clearly a potential
400 for substantial divergence in both nuclear and mitochondrial nucleotide sequences with no loss
401 of mitonuclear compatibility. Moreover, given many of the nuclear gene products that function
402 in the mitochondrion also have functions outside of the mitochondrion (Burak *et al.*, 2013;
403 Blumberg *et al.*, 2014; Chatterjee *et al.*, 2016), evolutionary changes to such proteins could be
404 driven by selection that is not related to mitonuclear coadaptation. Conversely, numerous
405 examples now support the contention that mitonuclear incompatibilities leading to loss of fitness
406 could be brought about by a few key changes to either the mitochondrial or nuclear genotype
407 (Aledo *et al.*, 2014; Camus *et al.*, 2015). Indeed, single point mutations in nuclear and
408 mitochondrial genes have been shown to be the basis for mitochondrial dysfunction in
409 heterospecific hybrid crosses (Meiklejohn *et al.*, 2013).

410 To date, most studies on mitonuclear interactions have focused on protein–protein
411 interactions in OXPHOS complexes co-encoded by nuclear and mitochondrial genomes (e.g.

412 (Kwong *et al.*, 2012; Osada & Akashi, 2012; Zhang & Broughton, 2013; Havird *et al.*, 2015b).
413 However, the biochemical machinery that enables translation of mitochondrial genes has both
414 nuclear protein components and mitochondrial RNA components, namely tRNAs and rRNAs
415 (Fig. 2B, C). The mitochondrial-encoded RNA components must co-function with nuclear-
416 encoded proteins (Wallace, 2007; Bar-Yaacov *et al.*, 2012; Burton & Barreto, 2012; Sloan *et al.*,
417 2014). The replication and transcription of the mitochondrial genome also involves the
418 interaction of nuclear-encoded proteins with the mtDNA itself (Ellison & Burton, 2008, 2010a)
419 (Fig. 2D).

420 Epistasis may occur even when there is no physical interaction of gene products because the
421 coordinated function of mitochondrial and nuclear gene products depends critically on retrograde
422 (mitochondria to nucleus) and anterograde (nucleus to mitochondria) signalling (Moore &
423 Williams, 2005; Woodson & Chory, 2008; Clark, Alani & Aquadro, 2012; Monaghan &
424 Whitmarsh, 2015; Baris *et al.*, 2017). Such signalling requires that both genomes correctly
425 recognize and respond to signals from each other. It should also be noted that new types of
426 mitonuclear interactions are still being discovered; for instance, there is some evidence that mt
427 genomes encode small RNAs (Pozzi *et al.*, 2017) and several small peptides, such as humanin
428 and mitochondrial open reading frame of the 12S rRNA-c (MOTS-c), are encoded by the mt
429 genome (Lee, Yen & Cohen, 2013; Lee *et al.*, 2015). These newly discovered mitochondrial
430 products could play an important role in retrograde signalling. In sum, mitonuclear
431 incompatibilities can be caused by more than just compromised protein–protein interactions.

432

433 **(5) Theme 5: mitonuclear coadaptation is dependent on complex genotype x genotype x**
434 **environment interactions**

435 There is a tendency to catalogue mitonuclear gene combinations as either high or poorly
436 performing in terms of their phenotypic effects. However, the phenotype that results from any
437 given mitochondrial and nuclear genotype combination will also depend critically on the
438 environment. This view is supported by laboratory studies of invertebrate models, which have
439 confirmed that the performance associated with particular mitonuclear genotypes is routinely
440 contingent on the environmental context (Ellison & Burton, 2006; Dowling, Abiega & Arnqvist,
441 2007; Dowling *et al.*, 2010; Arnqvist *et al.*, 2010; Hoekstra, Siddiq & Montooth, 2013; Zhu,
442 Ingelmo & Rand, 2014; Mossman *et al.*, 2016; Willett & Burton, 2001, 2003). As an example, in
443 crosses between the fruit flies *D. melanogaster* and *D. simulans*, incompatibilities in the products
444 of mt and nuclear genes slow down larval development and reduce survival at 25°C but have no
445 effect on growth or survival at 16°C (Hoekstra *et al.*, 2013).

446 In addition, studies of spatial variation in mitochondrial haplotype distributions in
447 humans and other metazoans in their natural environments demonstrate that mutational patterns
448 at key protein-coding genes within the mtDNA sequence closely conform to patterns predicted
449 under a scenario of climatic adaptation (Mishmar *et al.*, 2003; Ruiz-Pesini, 2004; Balloux *et al.*,
450 2009; Cheviron & Brumfield, 2009; Quintela *et al.*, 2014; Silva *et al.*, 2014; Morales *et al.*,
451 2015; Camus *et al.*, 2017). Indeed, a growing view has emerged that environmental context
452 dependency in mitochondrial disease expression is likely to be common in humans (Mishmar *et*
453 *al.*, 2003; Wallace, 2005).

454 For example, emerging evidence supports a role for climatic adaptation in shaping
455 population frequencies of a mtDNA mutation (T3394C) associated with Leber's hereditary optic
456 neuropathy (LHON) in humans, a mitochondrial disease that causes blindness, and which is
457 associated with male biases in penetrance. Ji *et al.* (2012) reported that although T3394C has

458 arisen multiple times across the human mitochondrial phylogeny, it is highly enriched on
459 haplotypes that are common in high-altitude Asian populations (M9 haplotype in Tibet, and the
460 C4a4 haplotype in the Indian Deccan Plateau). Indeed, the T3394C variant is 22 times more
461 likely to be found at altitudes above 1500 m than among low-altitude Han Chinese populations.
462 Furthermore, functional analyses of transmitochondrial cybrid lines have shown that this
463 mutation causes reductions in mitochondrial complex I activity of between 7 and 28% when
464 expressed on the lowland BC4 and F1 Asian haplotypes, but no such reductions on the M9
465 haplotype (Ji *et al.*, 2012). This result, when coupled with the observations of spatial enrichment
466 of the T3394C variant in high-altitude populations, lends support to the suggestion that the
467 3394C mutation could well be adaptive at high altitudes, while pathological at low altitudes.

468 Finally, we note that it is likely that the outcomes of mitonuclear interactions will vary across
469 the sexes, although research into this contention is still in its relative infancy. The two sexes
470 represent very different environments in which mitochondria must function. For example, the
471 female gonads and gamete are metabolically quiescent relative to their male counterparts (Short,
472 1997; Vaught & Dowling, 2018), with the gametes exhibiting striking differences in both size
473 and mtDNA copy number. Because mitochondria are transmitted through the female lineage,
474 mitochondrial mutations that are male-harming can in theory escape the action of natural
475 selection. Such mutations can therefore increase and linger in populations and be fixed through
476 neutral mechanisms or even spread through positive selection if they confer fitness advantages to
477 females (Frank & Hurst, 1996; Gemmell, Metcalf & Allendorf, 2004; Beekman, Dowling &
478 Aanen, 2014). This ‘mother’s curse’ may be key to explaining why mitochondrial diseases such
479 as LHON exhibit much higher penetrance in males (Yen, Wang & Wei, 2006; Ventura *et al.*,
480 2007; Milot *et al.*, 2017). Experimental evidence has emerged to indicate that some of the

481 genetic variation that delineates naturally occurring mtDNA haplotypes in fruit flies (*D.*
482 *melanogaster*) may exhibit male biases in effects on key life-history traits tied to reproduction
483 and survival (Innocenti, Morrow & Dowling, 2011; Camus, Clancy & Dowling, 2012; Camus *et*
484 *al.*, 2015; Dowling, Tompkins & Gemmell, 2015; Immonen *et al.*, 2016; Camus & Dowling,
485 2018). Indeed, some of this mitochondrial genetic variation appears to be overtly sexually
486 antagonistic, augmenting female reproductive outcomes, at cost to males (Camus & Dowling,
487 2018). The studies of sex biases in mitochondrial genetic variation conducted to date, however,
488 have all compared the sex-specific fitness effects of mtDNA haplotypes when placed against
489 highly controlled nuclear backgrounds lacking segregating genetic variation. While providing
490 proof-of-concept, future studies will need to establish whether male biases in levels of
491 mitochondrial genetic variation underpinning key life-history traits are replicable across diverse
492 nuclear backgrounds. Nonetheless, other case studies supporting the mother's curse hypothesis
493 have come to light in flies (Patel *et al.*, 2016), mice (Nakada *et al.*, 2006), rabbits (Smith, Turbill
494 & Suchentrunk, 2010) and humans (Martikainen *et al.*, 2017) of mtDNA polymorphisms
495 exerting negative effects exclusively on male components of fertility (Vaught & Dowling, 2018).

496

497 **V. IMPLICATIONS AND OUTLOOK**

498 **(1) Implications for understanding speciation**

499 We strongly advocate consideration of mitonuclear interactions in future studies that seek to
500 understand species boundaries in natural populations. As discussed below, rapid advances in
501 fields such as population genomics and molecular modelling increasingly allow inferences to be
502 made about mitonuclear interactions in wild populations of diverse species. However, a deeper
503 understanding of the fitness consequences of these interactions generally demands manipulative

504 experiments, such as quantitative genetic crosses and mitochondrial respiration measurements.
505 The practical constraints of working with many vertebrate species such as wild bird populations,
506 which produce few offspring and will not breed in captivity, make such experiments highly
507 challenging if not impossible. It can therefore be instructive to consider the study of speciation in
508 animals, notably wild populations of invertebrates, for which such constraints have been
509 overcome.

510 The aforementioned splash pool copepod, *Tigriopus californicus*, may be the model
511 system in which the role of mitonuclear interactions has been most completely studied (Burton *et*
512 *al.*, 2013; Yang *et al.*, 2017; Barreto *et al.*, 2018). Many aspects of these studies have been
513 detailed in the above sections of this review. Here we point out two aspects of the biology of this
514 organism that set the stage for development of mitonuclear coadaptation. First, rates of mtDNA
515 substitution are high: Willett (2012) estimated that the rate of substitutions at synonymous sites
516 in *T. californicus* mtDNA is 55-fold higher than the rate in nuclear genes. Second, *T. californicus*
517 populations show strong population structure. Restricted gene flow among populations has
518 resulted in not only high levels of mtDNA divergence across populations, but also the
519 opportunity for uniquely coadapted nuclear genotypes among populations in response to the
520 extensive mtDNA divergence (Barreto *et al.*, 2018).

521 Numerous studies of mitonuclear interactions have taken advantage of the *Tigriopus*
522 system and these have been discussed within each of the themes developed above. The
523 importance of considering fitness variation across life stages (Theme 1) can at least partially be
524 addressed in this system by determining allelic frequencies of candidate genes in a sample of
525 newly hatched larvae to their frequencies in surviving adults from the same cohort. In an analysis
526 of the alleles of the cytochrome *c* gene (*cytC*; a nuclear gene encoding a protein essential to

527 OXPHOS function), Willett & Burton (2001) observed expected Mendelian genotypic ratios in
528 F2 hybrid larvae, but in adult animals from the same cross they observed that the ratios were
529 skewed in favour of combinations that restored the population-specific coevolved mitonuclear
530 genotypes. In addition to suggesting that *cytC* has coevolved with mitotype, this type of
531 experiment isolates the form of selection as larval-to-adult viability selection. In this particular
532 case, *in vitro* biochemical experiments and site-directed mutagenesis further verified the
533 functional coevolution of *cytC* with mitotype, and demonstrated that only a single amino acid
534 substitution was needed to change dramatically the functional interaction between a nuclear gene
535 and a mitochondrially co-encoded OXPHOS complex (Rawson & Burton, 2002; Harrison &
536 Burton, 2006) (Theme 4).

537 Although there are clear examples of protein–protein interactions underlying
538 mitochondrial dysfunction in *Tigriopus* hybrids, it is also clear that other types of interactions
539 likely contribute to mitonuclear incompatibilities (Theme 4). Ellison & Burton, (2010) found that
540 population mismatches between mtDNA and mitochondrial RNA polymerase resulted in reduced
541 mtDNA gene expression, and Barreto & Burton (2013*b*) found evidence for coevolution in
542 nuclear-encoded ribosomal proteins that interact with rRNA encoded in the mtDNA. Recent
543 work suggests that there is a genome-wide pattern of elevated rates of evolution among nuclear
544 genes known to interact with mtDNA or its gene products compared to nuclear genes lacking
545 those interactions

546 As discussed earlier, the impact of environmental variation on mitonuclear interactions is
547 often overlooked and can be substantial (Theme 5). Willett & Burton (2003) found that the
548 relative fitness of *cytC* genotypes not only depended on mitotype (see above), but also on the
549 thermal environment. The disruptive effect of population mismatches in mtDNA and

550 mitochondrial RNA polymerase cited above is accentuated under conditions of osmotic stress
551 when the energetic costs of osmoregulation require an upregulation of mitochondrial ATP
552 synthesis (Ellison & Burton, 2008a).

553 These studies with copepods clearly established the negative effects of incompatibilities
554 between mitochondrial and nuclear genes that arise when populations diverge in allopatry, and
555 the potential for such incompatibilities to disrupt gene flow among populations. The implications
556 for speciation are clear. What remains to be established is the relative importance of mitonuclear
557 interactions compared to other potential mechanisms for disruption of gene flow in the process of
558 speciation, and such broader insights will only come with a consideration of mitonuclear
559 interactions in studies of speciation.

560

561 **(2) Implications for best practices in mitochondrial replacement therapy**

562 It is now well established that the penetrance of numerous disease-conferring mtDNA mutations
563 is affected by a range of modifier alleles that lie within the nuclear genome (Taanman, 2001;
564 Bykhovskaya *et al.*, 2004; Ballana *et al.*, 2007; Davidson *et al.*, 2009; Luo, Hou, & Yang, 2013;
565 Wang *et al.*, 2015; Morrow & Camus, 2017). Thus, signatures of mitonuclear epistasis,
566 moderating disease penetrance and outcomes, are known to be common in human populations.

567 To date, discussion of the capacity for mitochondrial replacement therapy to lead to
568 compromised mitonuclear function has focused on (1) the anticipation of problems based on
569 understanding patterns of genetic variation and structure within and among human populations,
570 and (2) an assessment of the outcomes of mitochondrial replacement in animal models and
571 humans. A mitonuclear perspective that draws on insights from studies of natural non-human
572 populations should be helpful in developing best practices for performing mitochondrial

573 replacement techniques in humans. First, following Theme 1, full assessment of the outcome of
574 any combination of mitochondrial and nuclear genes can only come after a complete lifetime
575 because many of the negative consequences of mitonuclear dysfunction might be late-onset
576 diseases like Alzheimer's or Parkinson's diseases (Hudson *et al.*, 2014).

577 Moreover, researchers should be skeptical of attempts to draw inferences regarding the
578 potential for mitonuclear incompatibilities in humans from assessments of genome-wide
579 covariation between levels of mtDNA and nuclear divergence in the adult population. This is
580 evident from a recent study that attempted to assess the risk of negative outcomes of
581 mitochondrial replacement therapy in humans based on mitonuclear incompatibilities by
582 observing patterns of mitochondrial haplotype and nuclear introgression in humans (Rishishwar
583 & Jordan, 2017). The researchers used the whole mitochondrial and nuclear genome sequences
584 of 2054 "healthy adult" humans available through the 1KGP project (The 1000 Genomes Project
585 Consortium, 2015), which included "five major continental population groups", to assess the
586 possibility that there may be incompatibilities between some mt and N-mt genes between some
587 human populations. The rationale for the analysis was that, if individuals who carry nuclear
588 genes from one population and mitochondrial genes from another population can exist as healthy
589 adults, then admixture of nuclear and mitochondrial genes from divergent populations might not
590 present a substantial risk of mitonuclear incompatibilities in mitochondrial replacement therapy.

591 There are multiple potential problems associated with such inferences. Firstly, they do not
592 adequately consider the possibility of selection operating against mitonuclear genotypes at earlier
593 life stages (Theme 1). Assessment of healthy adults provides no insights into whether selection
594 had eliminated mitonuclear incompatibilities at earlier life stages in individuals not sampled.
595 Secondly, such inferences are based on genome-wide patterns of mitonuclear association and

596 divergence, and such broad-brushed approaches to assessing mitonuclear genetics overlook the
597 likely scenario that the relevant associations among nuclear genes will be limited to relatively
598 small numbers of key loci that interact with the mt genome (Theme 4). As we have discussed
599 above, mitonuclear incompatibilities might be underpinned by divergence at a small number of
600 sequence sites that are under strong selection for mitochondrial function. Finally, the statement
601 that subjects in the data set were healthy presents an untested assumption because no phenotypic
602 data are presented in the study. Ideally, one would try to link population genomic patterns of
603 mitonuclear genotypes to detailed measures of phenotype (including information on
604 mitochondrial function; Theme 2) (Morales *et al.*, 2015; Baris *et al.*, 2017).

605 While technically challenging, it is important that biomedical researchers seek formally to
606 test the capacity for mitonuclear interactions to affect the outcomes of mitochondrial replacement
607 therapy in non-human primate models, or in human oocytes themselves. In this regard, a recent
608 study by Hyslop *et al.* (2016) provides potential insights into a role of mitonuclear interactions in
609 shaping health outcomes of zygotes following mitochondrial replacement. They utilized a
610 technique known as pronuclear transfer, to move the pronuclei of fertilized zygotes to
611 enucleated donor embryos, thus creating the opportunity to place the diploid nuclear zygote
612 contributed by one male and female alongside a mitochondrial haplotype contributed by a
613 different female. The authors used this approach to create two types of zygotes. Autologous
614 zygotes were generated by removing and then returning the same pronuclei back into the same
615 zygotes. Autologous zygotes were therefore procedural controls, carrying identical mitonuclear
616 genotypes prior to and following the procedure. Heterologous zygotes were generated by
617 transferring pronuclei from one zygote to another, using donor and parental oocytes that differed
618 in their mtDNA haplogroups. The authors reported lower rates of blastocyst formation in

619 heterologous zygotes than autologous zygotes, raising the possibility that mitonuclear
620 interactions could be conferring negative effects in the early stages of embryogenesis. That said,
621 the capacity to draw clear inferences from the experiment was somewhat limited by the caveat
622 that creation of heterologous zygotes involved transferring pronuclei from vitrified to fresh
623 oocytes, or *vice versa*, while creation of the autologous zygotes did not.

624 Humans currently experience substantial gene exchange among most populations around the
625 globe, but this connectivity among human populations is a recent event in the history of *Homo*
626 *sapiens*. Throughout most of the history of our species, numerous human populations evolved
627 largely in isolation (Akey *et al.*, 2004; Bamshad *et al.*, 2004). A consideration of gene flow
628 among human populations is important because, in a panmictic population, segregation
629 perpetually breaks down genetic associations established by selection (Eyre-Walker, 2017). By
630 contrast, among isolated populations there is a much greater capacity for the evolution of unique
631 genetic associations (Barreto *et al.*, 2018). The isolation of many populations throughout human
632 evolution makes it plausible that mitonuclear incompatibilities might exist in the modern human
633 population.

634

635 **(3) The future will rely on integration of molecular, biochemical and ecological approaches**

636 Looking to the future, we advocate that integrative approaches are needed to understand the
637 molecular basis and fitness consequences of mitonuclear interactions (Sunnucks *et al.*, 2017).
638 Genotype-to-phenotype links can be developed by combining: (i) sequencing of mitochondrial
639 and nuclear genomes to detect sites of selection in populations; (ii) molecular modelling and
640 mapping to predict the effects of substitutions on protein structure, function, and interactions;

641 and, where feasible, (iii) respirometry and fitness measurements to infer consequences of
642 substitutions at mitochondrial, organismal, and population levels.

643 Molecular modelling is emerging as a particularly valuable tool to make predictions
644 about the effects of mitonuclear interactions on mitochondrial respiration (Grossman *et al.*, 2004;
645 Scott *et al.*, 2011). Driven by recent advances in structural biology, complete three-dimensional
646 structures are now available of the mammalian OXPHOS complexes (Tsukihara *et al.*, 1996;
647 Iwata, 1998; Fiedorczuk *et al.*, 2016; Zhu, Vinothkumar & Hirst, 2016) and the mammalian
648 respirasome supercomplex (Gu *et al.*, 2016; Wu *et al.*, 2016; Guo *et al.*, 2017; Davies, Blum &
649 Kühlbrandt, 2018). It is therefore possible to use these structures to analyse direct molecular
650 interactions between nuclear and mitochondrial gene products. Construction of homology
651 models of sequenced variants of OXPHOS subunits facilitates predictions about how
652 substitutions may affect structure, function, and interactions of OXPHOS complexes. Such
653 approaches have inferred climate-driven positive selection in mitochondrial-encoded Complex I
654 components in a range of animal taxa (Finch *et al.*, 2014; Garvin *et al.*, 2014; Caballero *et al.*,
655 2015). Structural mapping has also recently provided evidence of epistatic interactions between
656 mitochondrial-encoded and nuclear-encoded Complex I variants potentially under climate-driven
657 selection (Garvin *et al.*, 2016; Morales *et al.*, 2018). As we have noted, mitonuclear
658 incompatibilities need not involve direct interactions within multi-subunit complexes (Innocenti
659 *et al.*, 2011; Baris *et al.*, 2017). Nevertheless, the direct molecular interactions between nuclear
660 and mitochondrial gene products remain leading candidates as sites of mitonuclear
661 incompatibilities. In accordance with Theme 4, homology modelling is also an option to probe
662 protein–DNA and protein–RNA mitonuclear interactions (Bar-Yaacov *et al.*, 2015).

663 It is important to note, however, that molecular understanding of mitonuclear interactions
664 remains in its infancy. The complete atomic resolution of Complex I and the respirasome were
665 only recently resolved (Fiedorczuk *et al.*, 2016; Gu *et al.*, 2016; Zhu *et al.*, 2016; Guo *et al.*,
666 2017) and only low-resolution structures of metazoan ATP synthase have been published (Zhou
667 *et al.*, 2015). As a result, there is limited information from only a handful of model species about
668 the structure, function, and interactions of many OXPHOS subunits and their residues. Thus, it is
669 rarely justified to make detailed mechanistic inferences from molecular modelling, and any
670 predictions should be treated with caution until empirically tested (e.g. respirometry
671 measurements). We anticipate that future advances will increase the predictive power of
672 molecular modelling: higher resolution structures of the respirasome and ATP synthase;
673 improved understanding of how OXPHOS complex structure relates to function at a range of
674 levels and in specific environments; and development of molecular dynamics simulations for
675 these complexes that may reveal the sources of environmental interactions. In turn, such
676 approaches may allow screening for compatible mitonuclear interactions in mitochondrial
677 replacement therapy and help consolidate genotype-to-phenotype links in mitonuclear ecology.

678

679 **VI. CONCLUSIONS**

680 (1) Only in the last couple of decades has the significance of mitochondrial variation, and the
681 interactions of mitochondrial and nuclear genes, been incorporated into a conceptual framework
682 for understanding population structure and speciation.

683 (2) Only a minority of studies consider the potential effects of mitonuclear interactions when
684 assessing adaptation and the genetic basis for variation in individual performance.

685 (3) Interacting mitochondrial and nuclear genotypes are likely to play a key role in how
686 populations are structured, with implications for the process of speciation and for medical
687 therapies involving recombining mitochondrial and nuclear genotypes.

688 (4) The key question is how much of an overall effect will arise from mitonuclear interactions
689 *versus* interactions among nuclear genes, and this question can only be answered with a greater
690 research focus on mitonuclear interactions.

691 (5) Our growing understanding of the coevolution, coadaptation, and co-function of the products
692 of mitochondrial and nuclear genes in natural populations has established a set of themes that
693 should guide further research.

694 (6) The future lies in the integration of a mechanistic understanding of the biochemical and
695 biophysical consequences of mitochondrial and nuclear genotypes with population biology and
696 ecology.

697

698 **VII. ACKNOWLEDGEMENTS**

699 Australian Research Council (FT160100022 and DP170100165) and Hermon Slade Foundation
700 (HSF 15/2) to D.K.D., Australia Research Council (DE170100310) to C.G., NIH R01
701 GM118046 to D.B.S., and NSF DEB1551466 to R.S.B. The authors thank Paul Sunnucks for
702 useful discussions and Adam Eyre-Walker and an anonymous reviewer for making suggestions
703 for improving the manuscript. Author contributions: conceptualization, G.E.H. and D.K.D. ;
704 writing, G.E.H., D.K.D., D.B.S., J.C.H., R.S.B., C.G.; figures, J.C.H.

705

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1260 Table 1. Five themes in the study of mitonuclear interactions

- 1261 1. **Relentless selection for mitonuclear compatibility across ontogeny.** Mitonuclear
1262 interactions have fitness consequences at multiple stages of development, which may
1263 result in compounding effects in filtering out maladapted mitonuclear genotypes.
1264
1265 Prediction: the frequencies of mitochondrial (mt) and nuclear-encoded mitochondrial (N-
1266 mt) genes are predicted to change across life stages via selection for mitonuclear
1267 compatibility and functionality.
1268
- 1269 2. **Mitonuclear coadaptation is manifested in mitochondrial physiology.** The localized
1270 role of mt gene products within the mitochondria leads to the expectation that deleterious
1271 effects of maladapted mitonuclear genotypes will be mediated by changes in
1272 mitochondrial function.
1273
1274 Prediction: incompatibilities in coadapted sets of mt and N-mt genes will have effects
1275 targeted to the physiological and biochemical properties of mitochondria.
1276
- 1277 3. **Generational delays.** Mitonuclear incompatibilities may be shielded by dominance in
1278 the F1 generation and may be affected by sex linkage.
1279
1280 Prediction: the negative effects of novel combinations of mitochondrial and nuclear genes
1281 may not be evident until F2 and later generations.
1282
- 1283 4. **Mitonuclear incompatibilities need not involve protein–protein interactions or
1284 myriad substitutions.** Although most attention has focused on the protein–protein
1285 interactions that occur within oxidative phosphorylation (OXPHOS) complexes, there are
1286 many other arenas for mitonuclear interactions, including mitochondrial translation,
1287 transcription, and DNA replication. Single changes in mt or nuclear genes can also cause
1288 severe incompatibilities. Moreover, because many or most of the sequence changes that
1289 contribute to divergence in nuclear and mt genes may be neutral, the actual variants
1290 responsible for mitonuclear incompatibilities likely represent a small subset of total
1291 sequence change.
1292
1293 Prediction: mitonuclear incompatibilities can be caused by a small number of variants
1294 that need not change amino acid sequence and that may not be proportional to overall
1295 sequence divergence.
1296
- 1297 5. **Mitonuclear coadaptation is dependent on complex genotype × genotype ×
1298 environment interactions.** Mitochondrial function and physiology is highly context
1299 dependent, so the signatures of mitonuclear coadaptation are likely to be as well.
1300
1301 Prediction: the outcomes of genetic interactions between mitochondrial and nuclear
1302 genomes will be dependent on the genetic [*via* epistasis involving other mtDNA and
1303 nuclear single nucleotide polymorphisms (SNPs)], physiological (e.g. the sex in which
1304 the mtDNA is expressed) and abiotic environment.

1305

1306 **Figure Legends:**

1307 **Fig. 1.** Predicted organismal fitness, organelle function, and potential for maladapted
1308 mitonuclear genotypes during mitochondrial replacement therapy. Three possible mitochondrial
1309 donors are shown, yielding variable degrees of conceivable mitonuclear incompatibilities.
1310 Importantly, deleterious mtDNA mutations (shown in red) have *known* fitness consequences,
1311 while those resulting from mitonuclear incompatibilities are *predicted* and likely complex.

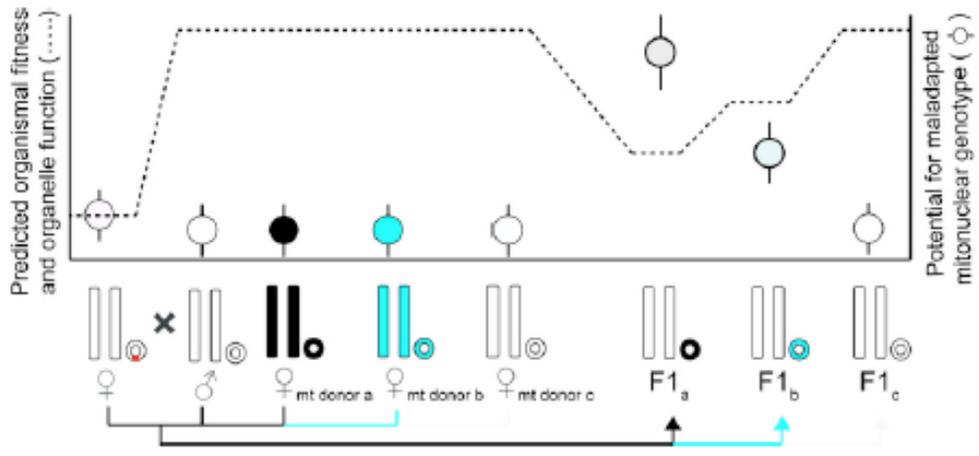
1312

1313 **Fig. 2.** Examples of mitonuclear interactions.: (A) multisubunit protein complexes of the electron
1314 transport chain, (B) mitochondrial ribosomal RNA (rRNA) and nuclear ribosomal proteins of the
1315 mitochondrial ribosome, (C) mitochondrial tRNA-Thr and nuclear threonyl-tRNA synthetase,
1316 and (D) mitochondrial DNA and nuclear DNA polymerase gamma. Non-interacting
1317 mitochondrial-encoded components are shown in green, nuclear-encoded components are in
1318 yellow, and interacting residues that physically contact residues encoded by both genomes are in
1319 red. All models are from mammals, except C which is from yeast. Interacting residues were
1320 identified following Sharbrough *et al.* (2017). PDB accessions used in structural depictions are
1321 5LNK, 1ZOY, 1BGY, 1V54, 5ARA, 3J9M, 4YYE, and 5C51.

1322

1323 **Fig. 3.** Predicted organismal fitness and organelle function across generations. In the F1
1324 generation, mitonuclear incompatibilities may generally be masked by retention of a maternal
1325 allele. Most mitonuclear incompatibilities are predicted to occur in F2 or later generations. After
1326 Burton *et al.* (2013).

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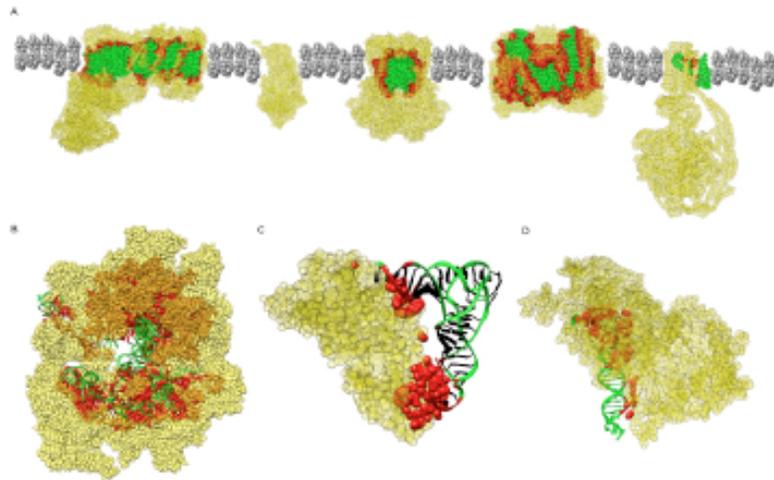
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1330 Figure 1

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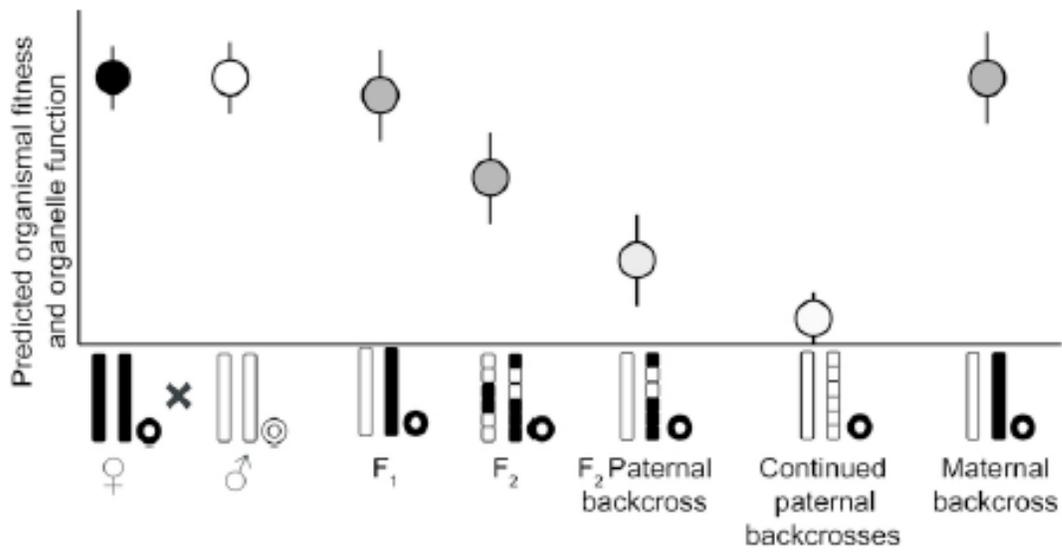
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1335 Figure 2

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