MONASH University

Theoretical and empirical insights into the ecology of oxidative phosphorylation in bilaterian metazoans

Tom Allison

A thesis submitted for the degree of Doctor of Philosophy at Monash University in 2023

School of Biological Sciences

Copyright notice

TABLE OF CONTENTS

Thesis abstract

The major process through which energy transduction occurs in modern organisms – oxidative phosphorylation (OxPhos) – is heavily regulated and highly refined. There are major differences, however, in the regulation and selective pressures that apply to oxidative phosphorylation in prokaryotes and eukaryotes. This is because, in eukaryotes, the genes controlling construction and regulation of the electron transport system upon which OxPhos relies are spread across two genomes. Furthermore, these genes may be subject to additional selective pressures stemming from obligate sexual reproduction, namely sexual selection. These differences may be particularly pronounced in bilaterian metazoans, which have evolved high metabolic demands. The evolutionary pathway toward optimisation of OxPhos in bilaterian metazoans is, therefore, likely subject to unique ecological constraints, and in turn likely produces unique solutions. In this thesis, I empirically explore the extent to which genetic control over OxPhos is shared across the two genomes; presenting results that suggest that additive effects of each genome influence OxPhos with greater magnitude compared to interactions between the two. Using a theoretical approach, I turn to unique ecological situations where interactions between the two genomes may dominate over additive effects, and show that a potential solution to optimising OxPhos function is the biparental inheritance of mitochondrial genomes. Finally, I assess whether OxPhos function is shaped by the strength of competition for mates, ultimately showing that the efficiency with which fuel and oxygen turned into usable energy evolves under variation in strength of sexual selection. Each of these pieces of work offer unique insights into complex shifts that have occurred in the ecology of OxPhos since its prokaryotic origins, and their consequences for modern bilaterian metazoans.

Publications during enrolment

Allison TM, Radzvilavicius AL, Dowling DK (2021) Selection for biparental inheritance of mitochondria under hybridisation and mitonuclear fitness interactions. *Proc. R. Soc. B.* **288**, 20211600

Thesis including published works declaration

I hereby declare that this thesis contains no material which has been accepted for the award of any other degree or diploma at any university or equivalent institution and that, to the best of my knowledge and belief, this thesis contains no material previously published or written by another person, except where due reference is made in the text of the thesis.

This thesis includes one original paper published in a peer reviewed journal and zero submitted publications. The core theme of the thesis is evolutionary biology. The ideas, development and writing up of all the papers in the thesis were the principal responsibility of myself, the student, working within the School of Biological Sciences under the supervision of Prof Damian K. Dowling and Prof Craig R. White

The inclusion of co-authors reflects the fact that the work came from active collaboration between researchers and acknowledges input into team-based research.

In the case of chapter II, my contribution to the work involved the following:

Thesis Chapter	Publication Title	Status (published, in press, accepted or returned for revision, submitted)	Nature and % of student contribution	Co-author name(s) Nature and % of Co- author's contribution*	Co-author(s), Monash student Y/N*
2	Selection for biparental inheritance of mitochondria under hybridization and mitonuclear fitness interactions	Published	Concept, theoretical model construction and analysis, writing first draft and editing (60%)	1) Damian Dowling: concept, drafting and editing (20%) 2) Arunas Radzvilavicius: model construction and analysis (20%)	No No

I have renumbered sections of submitted or published papers in order to generate a consistent presentation within the thesis.

Student name: Tom Allison

Student signature:

Date: 03/10/2023

I hereby certify that the above declaration correctly reflects the nature and extent of the student's and co-authors' contributions to this work. In instances where I am not the responsible author I have consulted with the responsible author to agree on the respective contributions of the authors.

Main Supervisor name: Damian K. Dowling

Main Supervisor signature:

Date: 03/10/2023

Acknowledgements

To Damian, who is the only person I know that can cover an entire word document with track changes, edits and corrections and still make you feel like the smartest person in the room. You are someone who cares as much about the people in science as the science itself, and for that I am so grateful. Working with you over the last five years has been a pleasure and an honour. I still get a kick out of seeing your name in the literature and realising that I get to work with one of the people who truly pioneered this field.

To Craig, I'm constantly blown away by the depth of your understanding of the practice and philosophy of science. Thank you for lending your hefty intellect and passion for evolutionary physiology on a weekly basis. Also, it's seriously inspiring to see someone bear fruit on a career spent focussed on a single, big question in biology. I can only hope to take a leaf from your book.

To Winston, Becca, Flo, the Dowling Lab and the UCL crew: in a very literal sense, I couldn't have done this work without you. Thank you for bringing my ideas to life, for the hours of fly work you helped me with and, Winston, for your red-hot memes.

Finally, to my wife: I love you, even if you don't love science. The happiness you get from me pursuing something I love – and the support you've given me along the way – is just one of the many reasons I'm lucky to spend my life with you.

CHAPTER I | General introduction

"Life is nothing but an electron looking for a place to rest"

Albert Szent-Györgyi

1.1 | THE ELECTRON TRANSPORT SYSTEM AND OXIDATIVE PHOSPHORYLATION

To grow and reproduce, organisms must extract free energy from their environment. In almost all life on earth, this feat is achieved via the creation and relaxation of a chemical gradient across phospholipid membranes (Mitchell 1961). In even the earliest cellular life, molecular mechanisms were in place that allowed organisms to facilitate the exergonic transfer of electrons from 'donor' molecules to 'accepter' molecules, and use the energy released in the process to move hydrogen ions across organic membranes (Mulkidjanian *et al* 2007; Lane *et al* 2010; Weis *et al*. 2016). The hydrogen ions, which don't easily pass through the membrane, build up on one side and begin to form a chemical disequilibrium across the membrane. Energy is stored in this 'chemiosmotic gradient' until hydrogen ions are permitted to flood through a large, membrane-bound protein (ATP synthase), which in turn uses the energy released to power the endergonic addition of an inorganic phosphorous group to adenosine diphosphate to form adenosine triphosphate – the energetic 'currency' of life.

The coupling of electron transfer reactions (oxidation-reduction reactions, or simply 'redox' reactions) to the storage of energy in the phosphoanhydride bonds of ATP via a chemiosmotic gradient is termed oxidative phosphorylation (OxPhos). Today, much is known about the finer mechanical and chemical details of oxidative phosphorylation and the structure upon which the process occurs - the electron transport system (ETS). Electrons most commonly enter the ETS by transfer from the electron donor NADH to a large, multi-unit, membrane-bound protein called Complex I (also known as NADH dehydrogenase). Within Complex I, electrons are passed between a series of 'iron-sulfur' clusters, with

Complex I harnessing the free energy released at each transfer and using it to 'pump' four hydrogen ions across a phospholipid membrane. Electrons may alternatively enter the ETS by transfer from $FADH_2$ to Complex II (or succinate dehydrogenase). Regardless of whether electrons enter the ETS at Complex I or Complex II, they are ultimately passed to Ubiquinone (or Co-enzyme Q), which acts as an electron shuttle within the membrane lipid-bilayer. Ubiquinone passes electrons to Complex III, which in turn passes them along to Complex IV (Cytochrome c Oxidase or simply 'COX'), and again uses the energy released to pump another four hydrogen ions across a membrane. Complex IV transfers electrons to the ultimate electron acceptor of the system – oxygen – whilst using the energy released to pump a further two hydrogen ions.

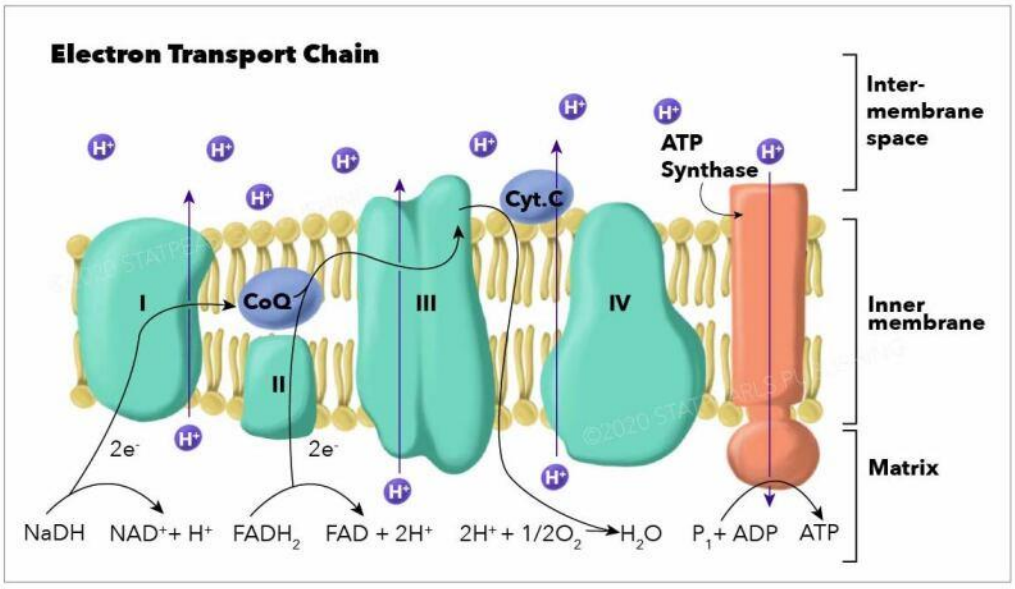

Figure 1. The electron transport system, showing electrons moving from complex I and complex II to complex III through CoQ, and then to complex IV, where they are passed to oxygen, producing water as a by-product. At each step, hydrogen ions are pumped from the mitochondrial matrix to the inter-membrane space, before they return to the matrix via ATP synthase, which fixes an inorganic phosphate to ADP to produce ATP.

In prokaryotes, the electron transport system is located within the cellular membrane, and protons are pumped from within the cytosol to the space enclosed by the membrane and the cell wall. In eukaryotes, however, the ETS is located within the inner membrane of the mitochondrion. In this system, protons are pumped from within the 'matrix' to the intermembrane space (between the inner and outer mitochondrial membranes). The parallels between the structure and function of the electron transport system in prokaryotes and mitochondria is unlikely to be a case of convergence. Rather, it is a clue to the origins of mitochondria. It is thought that mitochondria are modern vestiges of a long-ago symbiotic merger between an archaeal host and an alpha-proteobacteria symbiont (Margulis 1967; Martin & Muller 1998; Ryan & Hoogenraad 2007; Bar-Yaacov *et al*. 2012). In fact, some have gone on to argue that this merger defined the origin of eukaryotes (Martin *et al*. 2015; Lane 2015). By providing orders of magnitude increases in capacity to produce energy, early mitochondria permitted their hosts to explore a kind of morphological state space that was previously energetically inaccessible (Lane & Martin 2010). Thus, as the symbiosis between the two previously free-living prokaryotes persisted, each underwent drastic bioenergetic, genetic and morphological changes.

For the purposes of this thesis, I will draw attention to two key changes in the natural history of the electron transport system and the process of oxidative phosphorylation that have occurred following the genesis of eukaryotes and that have persisted in modern eukaryotes. The first is that the genes controlling ETS structure and OxPhos function became split across two distinct genomes: the nuclear and the mitochondrial genomes. By virtue of this distributed genomic control, groups of ETS protein coding genes that had been

inherited together and experienced selection as (more-or-less) a unit for nearly ~2 billion years were thrust into linkage equilibrium. Furthermore, as reproductive strategies evolved and cells began to specialise, genes controlling OxPhos found themselves associated with different 'mating types' that each had different paths to optimising evolutionary fitness (Speijer 2016). As this gametic asymmetry led into sexually reproducing bilaterian metazoans with high energetic demands and well-developed nervous systems capable of detecting a vast array of signals that may inform reproductive decisions, the genes controlling OxPhos became potential targets for sexual selection. The ecology, selective pressures, and control of the ETS in eukaryotes are apparently very different from the ETS in prokaryotes. This thesis is not intended to address the consequences of these ecological shifts across all eukaryotes in their entirety. Rather, it is an acknowledgement that, regarding OxPhos, some of the consequences of the eukaryotic merger have persisted into bilaterian metazoans, and require empirical investigation. How is variation in OxPhos function partitioned across the mitochondrial and nuclear genomes? Is variation in OxPhos shaped by sexual selection? Given dual genomic control of OxPhos, might we expect to see mechanisms in place that assist with forming co-adapted 'mito-nuclear' genetic combinations?

1.2 | OXPHOS FUNCTION UNDER DUAL GENOMIC CONTROL

An enduring consequence of the symbiotic merger that gave rise to eukaryotes is that OxPhos is now under the regulatory control of genes from two distinct genomes. During the early stages of the symbiotic merger, the alpha-proteobacteria symbiont – precursors of modern mitochondria – began to rapidly shed genes from their genome; perhaps due to the

selective advantage associated with more rapid replication of a smaller genome (Gray 2012; Kleine et al. 2009; Burger et al. 2003). Many of these genes were integrated into the growing nuclear genome of the proto-eukaryote (Adams & Palmer 2003; Kleine *et al*. 2009). In most modern bilaterian metazoans, only 37 genes remain in the circular, non-recombining genome of the mitochondrion: thirteen genes encode proteins that contribute to construction of all complexes in the ETS (with the notable exception of complex II, which is entirely nuclear-encoded), 22 encode transfer RNAs which are involved in protein translation and metabolic regulation, and finally two encode ribosomal RNA which directly assist with production of proteins within the mitochondrion (Wolstenholme 1992; Taanman 1999).

Given the abundance of mitochondrial related genes that were incorporated into the nuclear genome (Lotz *et al*. 2014), it is perhaps unsurprising that nuclear genetic variation is associated with variation in OxPhos function (Jumbo-Lucioni *et al*. 2012). Regarding the mitochondrial genome, however, theory predicted that very little functional variation; that is, genetic variation that leads to phenotypic consequences, should exist. It was thought that the importance of mitochondrial function to organismal survival should subject mtDNA to strong purifying selection that would reduce or eliminate genetic variation (reviewed in Ballard & Whitlock 2004; Galtier *et al*. 2009). Contrary to this prediction, mitochondrial genomes have been shown to exist in variants that *do* induce phenotypic variation (Ruiz-Pesini *et al*. 2004; Meiklejohn *et al*. 2007; Ballard & Kreitman 1995; Wallace *et al*. 1999; Ballard & Whitlock 2004; Rand *et al*. 2004; Dowling *et al*. 2008; Dobler *et al*. 2014). Variation in mtDNA has been linked to a range of traits, including key fitness components such as

longevity and fecundity (Aw *et al*. 2011, Camus *et al*. 2015, Camus & Dowling 2018; James & Ballard 2003; Maklakov et al. 2006; Camus *et al*. 2012).

MtDNA variation also affects OxPhos function, though the expression of these effects may depend on ecological factors and physiological states such as sex and age (Katewa & Ballard 2007; Pichaud *et al*. 2012, Correa *et al*. 2012, Pichaud *et al*. 2013, Fetterman *et al.* 2013, Wolff *et al*. 2016, Camus *et al*. 2023). It has been proposed that the nuclear genome may be one of the most important moderators of the effects of mitochondrial haplotype on OxPhos function (Rand *et al*. 2004; Dowling *et al*. 2008; Hill 2015; Dowling & Wolff 2023). The ETS is constructed as a mosaic of proteins; some encoded by nuclear and some by mitochondrial genes. Mitochondrial-encoded proteins adjoin nuclear-encoded proteins in four of the five complexes of the ETS, each with active sites that require fine-tuning to the spatial scale of angstroms (Efremov *et al*. 2010; Hirst 2013; Formosa & Ryan 2018). Thus, it is theorised that variation in either nuclear-encoded ETS proteins *or* mitochondrial-encoded ETS proteins may result in significant variation in OxPhos function. Accordingly, researchers have drawn attention to the possibility of epistatic interactions between the two genomes affecting OxPhos function (Rand *et al*. 2004; Dowling *et al*. 2008; Hill 2015). If selection on minimising errors in the OxPhos machinery is strong, epistatic interactions should lead to co-evolution between the two genomes. In line with predictions, we see that OxPhos function can be compromised by pairing mitochondria with nuclear genomes of distantly related species in mitonuclear hybrid cells. This has been demonstrated for both human-chimpanzee and rat-mouse mitonuclear hybrids (Barrientos *et al*. 1998; Dey *et al*. 2000). More recently, cross-breeding experiments using distinct populations of the intertidal copepod *Tigriopus californicus* have demonstrated that oxygen consumption at enzymatic complexes of the

ETS that are constructed with protein products from both genomes (CI and CIV) is reduced, but oxygen consumption is unaffected at complexes entirely constructed with nuclear encoded proteins (CII) (Ellison & Burton 2006). Similar results have been found for hybrid crosses of fruit-flies, with crosses between distinct combinations of *Drosophila mauritiana, Drosophila simulans* and *Drosophila melanogaster* yielding varying forms of bioenergetic dysfunction (Sackton *et al*. 2003; Pichaud *et al*. 2019). Furthermore, mutations in mtDNA that compromise OxPhos function in *D. simulans* can be functionally restored when paired with alternative nuclear genomes (Pichaud *et al*. 2019).

Further evidence for functional mitonuclear epistatic effects on fitness come from molecular studies, which reveal a higher-than-average ratio of non-synonymous to synonymous mutations (dN/dS) in nuclear genes that are related to OxPhos function (Blier *et al*. 2001). Importantly, the substitution rate in mitochondrial-related nuclear genes is higher than other nuclear genes and is comparable to the high substitution rate reported for mitochondrial genomes (Ballard & Whitlock 2004; Rand *et al*. 2004). This evidence furnishes support for a model whereby functional mutations in mtDNA place strong selective pressures on the mitochondrial-related nuclear genome to 'compensate' for the mutations. Researchers have since linked phylogenetic data to changes in protein structure in Complex IV of the ETS and showed that structural changes to mtDNA encoded proteins tended to precede structural changes to nuclear-encoded proteins (Osada & Akashi 2012). A similar pattern of correlated evolution has been observed between mitochondrial and nuclear genes encoding ribosomal RNAs at interacting sites in the mitochondrial ribosome (Barreto & Burton 2013). In aggregate, these findings suggest that selection for functional 'pairs' of

mitochondrial and nuclear alleles is persistent across metazoans, leading to lineages each with their own co-evolved mitonuclear 'matching set'.

Although evidence suggests that nuclear compensatory substitutions are likely widespread, the consequences of disrupting co-evolved pairs of alleles may still depend on factors such as divergence between populations from which mitochondrial and nuclear alleles are sourced. In recently diverged populations, with limited inter-population variation, additive effects of mtDNA may in fact supersede mitonuclear epistasis in terms of effect size. This is because the majority of substitutions in mtDNA are thought to be only mildly deleterious, with a significant proportion thought to be adaptive (Eyre-Walker & Keightley 2009; James *et al*. 2016; Morales *et al*. 2018). Under the assumption that deleterious mtDNA substitutions would place stronger selective pressures on the nuclear genome to compensate than adaptive mtDNA substitutions, the phenotypic consequences of disrupting co-evolved pairs may depend on the magnitude of effect sizes associated with the adaptive versus the deleterious substitutions. If the adaptive substitutions are large in magnitude, and deleterious substitutions smaller in magnitude, mtDNA main effects may outweigh epistatic effects despite the greater abundance of deleterious substitutions. However, when combinations of mitochondrial and nuclear alleles are sourced from greatly diverged populations, the abundance of nuclear compensatory changes (even if, in isolation, each is mild in its effect) may lead to a greater opportunity and magnitude of Bateson-Dobzhansky-Muller incompatibilities and therefore mitonuclear epistasis may predominate (Burton & Barreto 2012; Burton 2022).

1.3 | MECHANISMS THAT FACILITATE OPTIMAL MITONUCLEAR MATCHING

The partitioning of control over the OxPhos system across two distinct genomes has rendered the problem of minimising error in the system considerably more complex than if the system was encoded by one genome only. We may therefore expect that mechanisms or processes promoting fixation of beneficial combinations of alleles across the two genomes should be favoured by natural selection. One such process is thought to be the uniparental inheritance of mitochondria. In almost all eukaryotes, mitochondria and the associated mtDNA is inherited from only the female parent (Birky 2001). This unusual inheritance pattern is enforced by a range of highly specific biochemical machinery evolved to carry out either male-driven mitochondrial self-destruction in sperm or female-driven spermicide before or shortly after fertilisation (Chacko *et al*. 2019; DeLuca & O'Farrell 2012; Nishimura *et al*. 2020; Sutovsky *et al*. 2000). Uniparental inheritance reduces intracellular variance and increases between-cell variance in mtDNA (Radzvilavicius 2021), a pattern that is thought to yield several beneficial consequences. Theoretical work suggests that UPI may facilitate more rapid purification of harmful alleles, including any 'selfish' mtDNA mutants that may arise (Cosmides & Tooby 1981; Hoekstra 1990; Radvilavicius 2017). Secondly, it may allow more rapid fixation of beneficial alleles within populations (Christie & Beekman 2017; Radzvilavicius 2021). Together, these benefits of UPI have rendered it a system that has been favoured by natural selection – possibly repeatedly throughout the evolution of eukaryotes (Radzvilavicius *et al*. 2017).

Though uniparental inheritance of mitochondria is the dominant form of mitochondrial inheritance, it appears that there are patterns in the exceptions. Specifically, bi-parental inheritance of mitochondria (BPI) is not only present but persistent in many hybridising

populations (Kvist *et al*. 2003; Welch *et al*. 2006; Pearl *et al*. 2009; Morgan *et al*. 2013; Radojicic *et al*. 2015; Mastrantonio *et al*. 2019a; Mastrantonio *et al*. 2019b; Packert *et al*. 2019). In parallel, some of the most dramatic effects on OxPhos to date have been observed in hybrids of distantly related populations or experimentally produced mitonuclear hybrids (Ellison & Burton 2006; Ellison & Burton 2008, Niehuis et al 2008, Lee et al. 2008; Spirek et al 2014; Lamelza & Ailion 2017; Barrientos *et al*. 1998; Dey *et al*. 2000). Through an adaptationist lens, it remains a possibility that – at least in highly diverged hybridising populations – BPI could be another process that assists in optimising the 'match' between nuclear and mitochondrial alleles that have co-evolved in respective populations.

1.4 | PARTITIONING EFFECTS OF NUCLEAR AND MITOCHONDRIAL GENOMES ON OXPHOS – WHEN IS THE INTERACTION IMPORTANT?

If mechanisms that facilitate fit combinations of mitochondrial and nuclear alleles are to be favoured by selection only when mitonuclear epistasis affects fitness, it is important to understand the kind of genetic divergence between mitochondrial and nuclear genomes that, when paired, precipitates interactive effects on OxPhos function. Perhaps the most relevant level of genetic divergence to assess is the kind of genetic divergence routinely observed in wild, interbreeding populations. The implication of previous studies identifying strong molecular signals of mitonuclear coevolution is that even small mtDNA mutations are likely to cause large enough phenotypic changes to exert selective pressures on the nuclear genome. If mtDNA mutations commonly exert strong enough selective pressures on nuclear genomes to 'compensate', we may then expect that the performance of a given mitochondrial haplotype should be sensitive to the nuclear background with which it is

paired. In other words, we would expect to observe strong epistatic effects on OxPhos function even when pairing mitochondrial haplotypes with nuclear backgrounds of closely related populations (assuming that the different populations harbour different mtDNA haplotypes and are not connected by appreciably high levels of gene flow).

The model described above, whereby links between mtDNA sequence and OxPhos function depend sensitively on nuclear genome sequence, may be contrasted to another model whereby mtDNA sequence variation determines OxPhos function regardless of the nuclear genome with which it is paired. In support of the latter model, researchers frequently observe genealogical discordance between mitochondrial haplotypes and nuclear genotypes (Riesenberg & Soltis 1991; Currat *et al*. 2008; Funk & Omland 2003; Toews & Brelsford 2012). Such empirical findings may reflect the existence of mtDNA haplotypes that increase organismal fitness when paired with several nuclear backgrounds, rather than a unique, coevolved nuclear genotype (Sloan *et al*. 2017). This would diminish the importance of the mitonuclear interactive effect and suggest a larger role for mtDNA variation to act in an additive fashion. In reality, mtDNA sequence variation may affect organismal function in both an additive, and epistatic fashion. The fit of either model may be case-specific, depending in some way on the genetic divergence across breeding populations and the trait under selection.

To assess the importance of mitonuclear interactive effects, researchers have used experimental designs that allow them to detect phenotypic changes attributable to nuclear and mtDNA additive variation as well as their interaction. Whereas early studies created novel combinations of mtDNA and nuclear genotype derived from greatly diverged

(interspecific) lineages, more recent approaches have combined mitochondrial and nuclear genotypes from populations exhibiting far less divergence. Many of these studies have found that the mitonuclear interaction influences many traits including development rate, longevity, and physical performance (though there have been mixed results when assaying key determinants of fitness such as fecundity and reproductive success) (Clark & Lyckegaard 1988; Rand et al. 2001; Dowling et al. 2007; Montooth et al 2010; Immonen et al. 2016a; Camus et al. 2020; Rank *et al*. 2020; Immonen *et al*. 2016b; Rand et al 2006, Camus et al. 2012; Meiklejohn et al. 2013; Dong et al 2019; Carnegie et al. 2021; Mossman et al 2016 a,b; Sujkowski et al 2019). Relatively few of these studies focus on partitioning variance across additive *and* interactive effects (although see Dobler *et al.* 2014 and Dobler *et al*. 2018 for meta-analyses that attempt to partition effects attributable to each genome and their interaction across a range of traits). Indeed, the overwhelming amount of support for the existence of mitonuclear epistasis should not be taken as an indication that mtDNA variation doesn't contribute to phenotypic variation in an additive fashion. I argue that the goal of researchers should be to understand how important the interactive effect is *compared to* nuclear and mitochondrial genetic effects.

1.5 | OXPHOS UNDER SEXUAL SELECTION

The evolution of eukaryotes was intimately linked to the evolution of sexual reproduction: structured nuclear chromosomal recombination and fusion of nuclear haploid genomes to produce the next generation of diploid offspring (Cavalier-Smith 2010; Ramesh *et al*. 2005; Speijer *et al*. 2015). Following the evolution of sex was the evolution of different sized gametes (anisogamy) which, as a result, possessed distinct paths to evolutionary fitness

(Trivers 1972; Kokko & Jennions 2008). In many bilaterian metazoans, these differential strategies were frozen into place with the evolution of obligate sexual reproduction. This system of reproduction opened the possibility that organisms could fail to reproduce by not securing a mate. This allowed for competition in securing a mating partner and, in doing so, opened a new dimension of selection upon eukaryotes: sexual selection (Darwin 1871). Sexual selection has been defined as "the differences in reproduction that arise from variation among individuals in traits that affect success in competition over mates and fertilizations" (Andersson 1994). The range of behaviours that are encompassed by this definition can be partitioned into pre-copulatory and post-copulatory sexual selection. The former can be further grouped according to intrasexual competition wherein members of one sex – usually males – fight, posture or compete for access to reproducing females, and intersexual competition, wherein members of one sex – usually females – select members of the other sex according to some criteria. The latter can also be grouped according to intrasexual competition (often referring to competition between sperm from different males) and intersexual selection (or 'cryptic' female choice, where sperm may be stored in the reproductive tract and later either accepted or rejected). Regarding pre-copulatory intersexual selection (hereafter simply referred to as 'female choice'), theory posits that females may increase their inclusive fitness by choosing males that brandish accurate signals of 'good genes'; that is, those that increase the probability of their offspring surviving and reproducing (Hamilton & Zuk 1982; Kirkpatrick & Ryan 1991; Zahavi 1975)

If the ability to produce energy efficiently and voluminously is indeed a major determinant of fitness, then traits representing high metabolic capacity may signal genetic quality and be favoured under sexual selection (Hill & Johnson 2013; Hill 2018; Koch *et al*. 2017). In support

of this idea, researchers have historically found strong links between energy expenditure and display of sexual signals (reviewed in Kotiaho 2001). More recently, researchers have used experimental evolution to demonstrate that metabolic rate evolves under differences in sexual selection strength (Garlovsky *et al*. 2022)

I suggest, herein, that a previously ignored but important distinction exists between increased 'metabolic capacity' and OxPhos function. This is because increased energetic demands can be accommodated by a range of adaptive changes other than increased flux through individual ETS complexes. These include modifications to vasculature, oxygen carrier molecule density, mitochondrial density, mitochondrial morphology, and mitochondrial network dynamics (Clausen 1977; Green *et al*. 2017; Mairbauri 2013; Hochachka *et al*. 1996; Jornayvaz & Shulman 2010; Cogliati *et al*. 2016; Liesa & Shirihai 2013). Increased electron flux through the ETS is just one way of many that organisms can increase available supply of ATP. Selection for higher 'metabolic capacity' to support increased display of sexually selected traits will not, by necessity, select for increased electron flux through the ETS or higher efficiency in producing ATP from oxygen and reducing equivalents (assuming limited caloric intake). Thus, I argue, empirical assessment of OxPhos function under variation in sexual selection strength is warranted.

There are grounds to believe that OxPhos function, independent of whole-animal metabolic rate, is a trait that may be exposed to, and shaped by, sexual selection. For any given caloric intake, increased efficiency should generate greater ATP yields and increased allocation to energetically expensive tasks. Accordingly, high bioenergetic efficiency has been associated with higher growth rates in frogs and fish (Salin *et al*. 2012; Salin *et al*. 2019) and plumage

colouration in the house finch (Hill *et al*. 2019). These empirical associations between bioenergetic efficiency and individual organismal performance have given support to the idea that traits linked directly to OxPhos function may present as an honest signal of 'quality' or 'condition' (Hill & Johnson 2013). In fact, it has been suggested that the ability to efficiently maintain cellular processes through bioenergetic pathways is *the* defining criteria upon which choosy females may make their choice (Hill 2011). If variation in bioenergetic parameters governs individual performance in sexually selected traits, such as male competitiveness or ornament display, and/or females have evolved to prefer such signals, we may expect that experimentally altering the strength of sexual selection over multiple generations may lead to consistent differences in OxPhos function.

1.6 | STATEMENT OF RATIONALE

I sought to address several major outstanding questions surrounding the control and ecology of the electron transport system and OxPhos function in bilaterian metazoans.

My first aim was to understand whether bi-parental inheritance of mitochondria could be, under specific circumstances, an adaptive *process* that facilitates inheritance of optimal combinations of mitochondrial and nuclear alleles. This work is embedded within the greater aim – beyond the scope of the present thesis – of fully cataloguing the range of possible processes that facilitate the difficult control problem of minimising errors in a system controlled by two separate genomes that are inherited in loose linkage disequilibrium. Uniparental inheritance of mitochondria has received much academic attention as one such process that served to ensure optimal mitochondrial function

(Cosmides & Tooby 1981; Hoekstra 1990; Radzvilavicius 2022). Though UPI remains the dominant mode of mtDNA inheritance, there is a growing recognition that UPI is not universally adaptive, with specific ecological scenarios instead favouring biparental inheritance of mitochondria (Breton *et al*. 2007; Kuijper *et al*. 2015; Breton & Stewart 2015; Radvilavicius *et al*. 2017). An important goal, more broadly, is to understand which scenarios are likely to lead to adaptive BPI. In doing so, we can better understand whether specific cases of BPI are likely adaptive or maladaptive. I address my first goal with a theoretical approach; beginning with the initial empirical observation that many cases of BPI have been observed in systems of hybridising populations. Hybridisation is known to frequently result in hybrid incompatibilities; a proportion of which may be mitonuclear incompatibilities (Gershoni *et al*. 2009; Burton & Barreto 2012). Specifically, the dual genomic control of OxPhos and the associated mito-nuclear compensatory co-evolutionary dynamic suggests that 'breaking up' putatively co-evolved pairs of mitonuclear alleles may result in OxPhos dysfunction (Rand *et al*. 2001; Dowling *et al*. 2008). Indeed, experimental evidence suggests that mixing mitonuclear combinations from greatly divergent lineages results in large dysfunction (Ellison & Burton 2006; Barrientos *et al*. 1998; Dey *et al*. 2000). While uniparental inheritance has been presented as the primary OxPhos error minimisation system in eukaryotes generally, I argue that a maladaptive case of UPI may not be the most parsimonious explanation for the presence and persistence of biparental inheritance of mitochondria in hybridising populations. There is a need for further theoretical investigation and modelling to understand the adaptive value of biparental inheritance under selection on mitonuclear interactions in hybridising populations – and therefore I have chosen to address it as my first thesis aim.

My second aim was to understand the relative influence of both mitochondrial and nuclear genomes, as well as their interaction, in shaping variation in OxPhos function. It is known that genes relevant to the ETS and, therefore, OxPhos, are housed in each of the genomes. It is unclear, however, how much functional variation (regarding OxPhos function) exists in each genome, and whether the functional variation within each genome depends on the other. Despite substantial experimental evidence for the impact of mitonuclear interactions on OxPhos under extreme sequence divergence (Ellison & Burton 2006; Barrientos *et al*. 1998; Dey *et al*. 2000), there are relatively few studies that aim to explicitly partition the relative contributions of the mitochondrial and nuclear genetic additive effects along with their interaction in study systems where the divergence across mitochondrial haplotypes is representative of that commonly segregating across interbreeding, wild populations. I argue that partitioning variance across the nuclear genome, mitochondrial genome, and their interaction, particularly with reference to variation in OxPhos function, will allow a greater understanding of how frequently we may expect to observe functional mitonuclear epistasis in wild populations and thus how important those interactions are to the evolution of those populations.

My third and final aim was to understand whether OxPhos function is shaped by sexual selection. If so, what are the bioenergetic parameters under selection and which direction does sexual selection guide them? Bilaterian metazoans, among the entire tree of life, are exceptional in their high energetic demands and extremely well-developed nervous systems. High energetic demands may place selective pressure on efficient energy production. And well-developed nervous systems may allow organisms to detect signals related to that energy production in potential mating partners, and base reproductive

decisions upon this information (Endler 1982; Uetz & Roberts 2002; Arnqvist 2006). The potential for organisms to recognise signals of metabolic function and base reproductive choices on this information has also been recognised in the literature (Buchanan *et al*. 2001; Hill *et al*. 2011; Koch *et al*. 2018). Furthermore, differences in metabolic phenotype have been linked to between individual variation in performance in a range of traits (Salin *et al*. 2012; Salin *et al*. 2019; Metcalfe *et al*. 2015; Auer *et al*. 2016; Heine & Hood 2020), which suggests that differences in metabolic capacity may allow for variation in peer-to-peer competitiveness with respect to dominating territory or mates. With almost universal obligate sexual reproduction across metazoans, it is possible that sexual selection can fine-tune bioenergetic parameters. Indeed, the links between whole animal metabolic rate and sexual selection have received attention (Berger *et al*. 2014; Garlovsky *et al*. 2022; Arnqvist *et al*. 2022). However, bioenergetic parameters and whole animal metabolism are two distinct traits that could respond independently to sexual selection. Thus, I argue, the links between OxPhos phenotype and sexual selection warrant investigation. Experimental evolution, where populations are exposed to enforced differences in strength of sexual selection over many generations, may offer a powerful way to test such links. No study has yet attempted to assess bioenergetic function in populations exposed for multiple generations to artificially enforced differences in the strength of sexual selection. Addressing this gap in the literature, therefore, became my third and final aim.

1.7 | SUMMARY OF CHAPTERS

1.7.1 | Chapter II

This chapter explores the possibility that bi-parental inheritance of mitochondria can be adaptive under hybridisation when we assume the presence of mito-nuclear interactions on fitness. I approach this problem from a theoretical perspective by creating and analysing a deterministic simulation of populations of single-celled organisms hybridising in a metapopulation. I take, as a starting assumption, the experimentally demonstrated notion that pairing mitochondrial and nuclear alleles from greatly divergent populations can result in OxPhos dysfunction and fitness decline. I then introduce a locus that controls mitochondrial inheritance mode, with two alleles: one that encodes strict uniparental inheritance and another, hypothetical allele that allows biparental inheritance of mitochondria when gametes containing nuclear genomes from distinct populations meet (and uniparental inheritance otherwise). With this model, I show that the 'mate-specific biparental inheritance allele' can invade the population to varying equilibrium frequencies depending on the level of gene flow throughout the metapopulation. I find that, particularly under higher levels of gene flow, strict uniparental inheritance greatly increases the likelihood of a zygote encounters the worst possible fitness outcome (a total 'mitonuclear mismatch') and that this risk is mitigated by the biparental inheritance strategy. I suggest that biparental inheritance of mitochondria in hybridising populations could act as a 'bet-hedging' or risk-avoidance strategy, whereby the benefits of highest fitness outcomes are traded off for avoidance of the lowest possible fitness outcomes.

1.7.2 | Chapter III

In this chapter, I attempt to partition the variance in OxPhos function according to nuclear and mitochondrial genetic variation, as well as their interaction. I approach this problem empirically by creating nine strains of fruit-fly (*Drosophila melanogaster*), each possessing orthogonal combinations of mitochondrial and nuclear genotype sourced from three allopatric populations of *D. melanogaster*. Crucially, the variation in protein-coding regions of mtDNA across these three populations is in the range of ~0.3%, which is representative of the kind of variation observed in wild, interbreeding populations (Morrow *et al*. 2015). I take high-resolution measurements of respiration in permeabilised fly thoraces, allowing us to assess oxygen consumption under various states of stimulation of the ETS. I find that variation in OxPhos can be accurately split along orthogonal axes representing 'coupled' (ATP-producing) and 'uncoupled' (non-ATP producing) respiration. In this system, both mitochondrial and nuclear genomes harbour variation that affects coupled respiration in an additive fashion. Furthermore, uncoupled respiration is subject to both nuclear genetic variation as well as an interaction between mitochondrial and nuclear genotypes. Importantly, I find that in each of the primary states of respiration, effect sizes associated with mitonuclear interactions are smaller in magnitude than either nuclear or mitochondrial main effects. These findings suggest that while mitonuclear interactions are likely a relevant selective force in most populations, additive mitochondrial genetic variation may be an underappreciated contributor to shaping population evolutionary trajectories.

1.7.3 | Chapter IV

In this chapter, I assess whether OxPhos function of lab-reared populations of *Drosophila melanogaster* may evolve in response to alterations in the strength of sexual selection. I

wanted to include all possible mechanisms of sexual selection including male-male competition, female choice as well as post-copulatory mechanisms of selection. Thus, I leveraged populations of *Drosophila melanogaster* that had been subjected to over 300 generations of either brief (1hr) or long (42h) mating windows. This difference in duration of male-female contact allowed for heightened male-male competition, stronger female choice as well as all forms of post-copulatory selection. I took high resolution measurements of respiration in permeabilised fly thoraces, allowing me to assess oxygen consumption under various states of stimulation of the ETS. I find that populations subject to higher sexual selection exhibit consistently higher respiratory control ratios, suggesting that these populations have more efficient conversion of oxygen and electron donors to ATP. This is the first demonstration that OxPhos function is subject to variation under strong and weak sexual selection.

1.8 | REFERENCES

Adams KL, Palmer JD (2003) Evolution of mitochondrial gene content: gene loss and transfer to the nucleus. Mol Phylogenet Evol. **29**, 380-95

Andersson M (1994) *Sexual Selection*. Princeton University Press, Princeton, NJ.

Arnqvist G (2006) Sensory exploitation and sexual conflict. *Philos Trans R Soc Lond B Biol Sci.* **361**, 375-86.

Arnqvist G, Rönn J, Watson C, Goenaga J, Immonen E (2022) Concerted evolution of metabolic rate, economics of mating, ecology, and pace of life across seed beetles. *Proc Natl Acad Sci USA.* **119**, e2205564119.

Auer SK, Salin K, Anderson GJ, Metcalfe NB (2016) Flexibility in metabolic rate and activity level determines individual variation in overwinter performance. *Oecologia* **182**, 703-712.

Aw WC, Correa CC, Clancy DJ, Ballard JW (2011) Mitochondrial DNA variants in Drosophila melanogaster are expressed at the level of the organismal phenotype. Mitochondrion **11**, 756-63.

Ballard JW, Whitlock MC (2004) The incomplete natural history of mitochondria. Mol Ecol. **13**, 729-744.

Ballard JW, Kreitman M (1995) Is mitochondrial DNA a strictly neutral marker? Trends Ecol Evol. **10**, 485-488.

Ballard JW, Melvin RG, Miller JT, Katewa SD (2007) Sex differences in survival and mitochondrial bioenergetics during aging in Drosophila. *Aging Cell* **6**, 699-708.

Barreto FS, Burton RS (2013) Evidence for compensatory evolution of ribosomal proteins in response to rapid divergence of mitochondrial rRNA. *Molecular Biology and Evolution* **30**, 310-314.

Barrientos A, Kenyon L, Moraes CT (1998) Human xenomitochondrial cybrids. Cellular models of mitochondrial complex I deficiency. *J Biol Chem*. **273**, 14210-7.

Bar-Yaacov D, Blumberg A, Mishmar D (2012) Mitochondrial-nuclear co-evolution and its effects on OXPHOS activity and regulation. Biochim Biophys Acta **1819**, 1107-1111.

Berger D, Berg EC, Widegren W, Arnqvist G, Maklakov AA (2014) Multivariate intralocus sexual conflict in seed beetles. *Evolution* **68**, 3457-69.

Blier PU, Dufresne F, Burton RS (2001) Natural selection and the evolution of mtDNA-encoded peptides: evidence for intergenomic co-adaptation. *Trends Genet* **17**, 400–406.

Birky CW Jr (2001) The inheritance of genes in mitochondria and chloroplasts: laws, mechanisms, and models. *Annu Rev Genet*. **35**, 125-48.

Breton S, Beaupré HD, Stewart DT, Hoeh WR, Blier PU (2007) The unusual system of doubly uniparental inheritance of mtDNA: isn't one enough? *Trends Genet.* **23**, 465-74.

Breton S, Stewart DT (2015) Atypical mitochondrial inheritance patterns in eukaryotes. *Genome* **58**, 423-31.

Buchanan KL, Evans MR, Goldsmith AR, Bryant DM, Rowe LV (2001) Testosterone influences basal metabolic rate in male house sparrows: a new cost of dominance signalling? *Proc Biol Sci.* **268**, 1337-44.

Burger G, Gray MW, Lang BF (2003) Mitochondrial genomes: anything goes. *Trends Genet.* **19**, 709–716.

Burton RS, Ellison CK, Harrison JS (2006) The sorry state of F2 hybrids: consequences of rapid mitochondrial DNA evolution in allopatric populations. *Am Nat.* **168**, S14-24.

Burton RS, Barreto FS (2012) A disproportionate role for mtDNA in Dobzhansky-Muller incompatibilities? *Mol Ecol*. **21**, 4942-57.

Burton RS (2022) The role of mitonuclear incompatibilities in allopatric speciation. *Cell Mol Life Sci*. **79**, 103

Camus MF, Clancy DJ, Dowling DK (2012) Mitochondria, maternal inheritance, and male aging. Curr Biol. **22**, 1717-1721.

Camus MF, Wolf JB, Morrow EH, Dowling DK (2015) Single Nucleotides in the mtDNA Sequence Modify Mitochondrial Molecular Function and Are Associated with Sex-Specific Effects on Fertility and Aging. Curr Biol. **25**, 2717-2722.

Camus MF, Dowling DK (2018) Mitochondrial genetic effects on reproductive success: signatures of positive intrasexual, but negative intersexual pleiotropy. *Proc Biol Sci*. **285**, 20180187.

Camus MF, Rodriguez E, Kotiadis V, Carter H, Lane N (2023) Redox stress shortens lifespan through suppression of respiratory complex I in flies with mitonuclear incompatibilities. *Exp Gerontol*. **175**, 112158.

Camus MF, O'Leary M, Reuter M, Lane N (2020) Impact of mitonuclear interactions on life-history responses to diet. *Philos Trans R Soc Lond B Biol Sci*. **375**, 20190416.

Cavalier-Smith T (2010) Origin of the cell nucleus, mitosis and sex: roles of intracellular coevolution. *Biol Direct* **4**, 5-7.

Chacko LA, Mehta K, Ananthanarayanan V (2019) Cortical tethering of mitochondria by the anchor protein Mcp5 enables uniparental inheritance. *J Cell Biol.* **218**, 3560-3571.

Christie JR, Beekman M (2017) Uniparental inheritance promotes adaptive evolution in cytoplasmic genomes. *Mol. Biol. Evol.* **34**, 677-691.

Clark AG, Lyckegaard EM (1988) Natural selection with nuclear and cytoplasmic transmission. III. Joint analysis of segregation and mtDNA in Drosophila melanogaster. *Genetics* **118**, 471-81.

Clausen JP (1977) Effect of physical training on cardiovascular adjustments to exercise in man. *Physiol Rev* **57**, 779-815.

Cogliati S, Enriquez JA, Scorrano L (2016) Mitochondrial Cristae: Where Beauty Meets Functionality. *Trends Biochem Sci* **41**, 261-273.

Correa CC, Aw WC, Melbin RG, Pichaud N, Ballard JWO (2012) Mitochondrial DNA variants influence mitochondrial bioenergetics in *Drosophila melanogaster*. *Mitochondrion* **12**, 459-464

Cosmides LM, Tooby J (1981) Cytoplasmic inheritance and intragenomic conflict. *J Theor Biol.* **89**, 83-129.

Currat M, Ruedi M, Petit RJ, Excoffier L (2008) The hidden side of invasions: massive introgression by local genes. *Evolution* **62**, 1908-20.

Darwin C (1871) *The descent of man, and Selection in relation to sex, Vol. 1.* John Murray.

DeLuca SZ, O'Farrell PH (2012) Barriers to male transmission of mitochondrial DNA in sperm development. *Dev Cell.* **22**, 660-8.

Dey R, Barrientos A, Moraes CT (2000) Functional constraints of nuclear-mitochondrial DNA interactions in xenomitochondrial rodent cell lines. *J Biol Chem*. **275**, 31520-7.

Dobler R, Rogell B, Budar F, Dowling DK (2014) A meta-analysis of the strength and nature of cytoplasmic genetic effects. *J Evol Biol*. **27**, 2021-34.

Dobler R, Dowling DK, Morrow EH, Reinhardt K (2018) A systematic review and meta-analysis reveals pervasive effects of germline mitochondrial replacement on components of health. *Hum Reprod Update*. **24**, 519-534.

Dong W, Dobler R, Dowling DK, Moussian B (2019) The cuticle inward barrier in *Drosophila* melanogaster is shaped by mitochondrial and nuclear genotypes and a sex-specific effect of diet. *PeerJ* **7**,e7802.

Dowling DK, Friberg U, Hailer F, Arnqvist G (2007). Intergenomic epistasis for fitness: within-population interactions between cytoplasmic and nuclear genes in Drosophila melanogaster. *Genetics* **175**, 235-44.

Dowling DK, Friberg U, Lindell J (2008) Evolutionary implications of non-neutral mitochondrial genetic variation. Trends Ecol Evol. **23**, 546-554

Dowling DK, Wolff JN (2023) Evolutionary genetics of the mitochondrial genome: insights from Drosophila. *Genetics.* **224**, iyad036.

Dobler R, Rogell B, Budar F, Dowling DK (2014) A meta-analysis of the strength and nature of cytoplasmic genetic effects. J Evol Biol. **27**, 2021-2034.

Efremov RG, Baradaran R, Sazanov LA (2010) The architecture of respiratory complex I. *Nature* **465**, 441-445

Ellison CK, Burton RS (2006) Disruption of mitochondrial function in interpopulation hybrids of Tigriopus californicus. *Evolution* **60**, 1382-91.

Ellison CK, Burton RS (2008) Interpopulation hybrid breakdown maps to the mitochondrial genome. *Evolution* **62**, 631-8.

Endler JA (1992) Signals, Signal Conditions, and the Direction of Evolution. *The American Naturalist **139**,* 125–153.

Eyre-Walker A, Keightley PD (2007) The distribution of fitness effects of new mutations. *Nat Rev Genet*. **8**, 610-618.

Fetterman JL, Zelickson BR, Johnson LW, Moellering DR, Westbrook DG, Pompilius M, Sammy MJ, Johnson M, Dunham-Snary KJ, Cao X, Bradley WE, Zhang J, Wei CC, Chacko B, Schurr TG, Kesterson RA, Dell'italia LJ, Darley-Usmar VM, Welch DR, Ballinger SW (2013) Mitochondrial genetic background modulates bioenergetics and susceptibility to acute cardiac volume overload. *Biochem J*. **15**, 157-67.

Formosa LE, Ryan MT (2018) Mitochondrial OXPHOS complex assembly lines. *Nat Cell Biol.* **20**, 511-513.

Funk DJ, Omland KE (2003) Species-level paraphyly and polyphyly: frequency, causes and consequences, with insights from animal mitochondrial DNA. *Ann Rev Eco. Evo & Sys.* **34**, 397-423.

Galtier N, Nabholz B, Glémin S, Hurst GDD (2009) Mitochondrial DNA as a marker of molecular diversity: a reappraisal. *Molecular Ecology* **18**, 4541-4550.

Garlovsky MD, Holman L, Brooks AL, Novicic ZK, Snook RR (2022) Experimental sexual selection affects the evolution of physiological and life-history traits. *J Evol Biol* **35**, 742-751.

Gershoni M, Templeton AR, Mishmar D (2009) Mitochondrial bioenergetics as a major motive force of speciation. *Bioessays* **31**, 642-50.

Gray MW (2012) Mitochondrial evolution. Cold Spring Harb Perspect Biol. **4**, a011403

Green DJ, Spence A, Rowley N, Thijssen DH, Naylor LH (2012) Vascular adaptation in athletes: is there an 'athlete's artery'? *Exp Physiol* **97**, 295-304.

Gutsaeva DR, Carraway MS, Suliman HB, Demchenko IT, Shitara H, Yonekawa H, Piantadosi CA (2008) Transient hypoxia stimulates mitochondrial biogenesis in brain subcortex by a neuronal nitric oxide synthase-dependent mechanism. *J Neurosci* **28**, 2015-24

Hamilton WD, Zuk M (1982) Heritable true fitness and bright birds: a role for parasites? *Science* **218**, 384-7.

Heine KB, Hood WR (2020) Mitochondrial behaviour, morphology, and animal performance. *Biol Rev Camb Philos Soc*. **95**, 730-737.

Hill GE (2011) Condition-dependent traits as signals of the functionality of vital cellular processes. *Ecol Lett* **14**, 625-34.

Hill GE, Johnson JD (2013) The mitonuclear compatibility hypothesis of sexual selection. *Proc Royal Society B* **280**, 20131314

Hill GE (2015) Mitonuclear Ecology. *Mol Biol Evol*. **32**, 1917-1927.

Hill GE (2018) Mitonuclear Mate Choice: A Missing Component of Sexual Selection Theory? *Bioessays* **40**.

Hill GE, Hood WR, Ge Z, Grinter R, Greening C, Johnson JD, Park NR, Taylor HA, Andreasen VA, Powers MJ, Justyn NM, Parry HA, Kavazis AN, Zhang Y (2019) Plumage redness signals mitochondrial function in the house finch. *Proc Biol Sci.* **286**, 20191354.

Hirst J (2013) Mitochondrial complex I. *Annu Rev Biochem*. **82**, 551-575.

Hochachka PW, Buck LT, Doll CJ, Land SC (1996) Unifying theory of hypoxia tolerance: molecular/metabolic defense and rescue mechanisms for surviving oxygen lack. *Proc Natl Acad Sci USA* **93**, 9493-8.

Hoekstra RF (1990) Evolution of uniparental inheritance of cytoplasmic DNA. In *Organisational constraints on the dynamics of evolution* (eds Maynard-Smith J, Vida J). Manchester, UK: Manchester University Press.

Immonen E, Collet M, Goenaga J, Arnqvist G (2016a) Direct and indirect genetic effects of sex-specific mitonuclear epistasis on reproductive ageing. *Heredity* **116**, 338-47.

Immonen E, Rönn J, Watson C, Berger D, Arnqvist G (2016b) Complex mitonuclear interactions and metabolic costs of mating in male seed beetles. *J Evol Biol*. **29**, 360-70.

James JE, Piganeau G, Eyre-Walker A (2016) The rate of adaptive evolution in animal mitochondria. *Mol Ecol.* **25**, 67-78.

James AC, Ballard JW (2003) Mitochondrial genotype affects fitness in Drosophila simulans. *Genetics* **164**, 187-94.

Jornayvaz FR, Shulman GI (2010) Regulation of mitochondrial biogenesis. Essays Biochem **47**, 69-84

Jumbo-Lucioni P, Bu S, Harbison ST, Slaughter JC, Mackay TFC, Moellering DR, De Luca M (2012) Nuclear genomic control of naturally occurring variation in mitochondrial function in *Drosophila melanogaster*. *BMC Genomics* **13**, 659

Kirkpatrick M, Ryan MJ (1991) The evolution of mating preferences and the paradox of the lek. *Nature* **350**, 33–38.

Kleine T, Maier UG, Leister D (2009) DNA transfer from organelles to the nucleus: the idiosyncratic genetics of endosymbiosis. Annu Rev Plant Biol. **60**, 115-138.

Koch RE, Josefson CC, Hill GE (2018) Mitochondrial function, ornamentation, and immunocompetence. *Biol Rev Camb Philos Soc* **92**, 1459-1474

Kokko H, Jennions MD (2008) Parental investment, sexual selection and sex ratios. *J Evol Biol* **21**, 919-48.

Kondo R, Satta Y, Matsuura ET, Ishiwa H, Takahata N, Chigusa SI (1990) Incomplete maternal transmission of mitochondrial DNA in Drosophila. *Genetics* **126**, 657-663.

Kotiaho JS (2001) Costs of sexual traits: a mismatch between theoretical considerations and empirical evidence. *Biol Rev Camb Philos Soc* **76**, 365-76.

Kuijper B, Lane N, Pomiankowski A (2015) Can paternal leakage maintain sexually antagonistic polymorphism in the cytoplasm? *J Evol Biol* **28**, 468-80

Kvist L, Martens J, Nazarenko AA, Markku O (2003) Paternal leakage of mitochondrial DNA in the Great Tit (*Parus major*). *Molecular Biology and Evolution* **20**, 243-247.

Lamelza P, Ailion M (2017) Cytoplasmic-nuclear incompatibility between wild isolates of caenorhabditis nouraguensis. *G3* **7**, 823-834.

Lane N, Allen JF, Martin WF (2010) How did LUCA make a living? Chemiosmosis in the origin of life. *BioEssays* **32**, 271-280.

Lane N, Martin WF (2010) The energetics of genome complexity. *Nature* **467**, 929–934.

Lane N (2015) *The Vital Question: Energy, Evolution, and the Origins of Complex Life*. W. W. Norton & Company.

Lee HY, Chou JY, Cheong L, Chang NH, Yang SY, Leu JY (2008) Incompatibility of nuclear and mitochondrial genomes causes hybrid sterility between two yeast species. *Cell* **135**, 1065-1073.

Liesa M, Shirihai OS (2013) Mitochondrial dynamics in the regulation of nutrient utilization and energy expenditure. *Cell Metab* **17**, 491-506.

Lotz C, Lin AJ, Black CM, Zhang J, Lau E, Deng N, Wang Y, Zong NC, Choi JH, Xu T, Liem DA, Korge P, Weiss JN, Hermjakob H, Yates JR 3rd, Apweiler R, Ping P (2014) Characterization, design, and function of the mitochondrial proteome: from organs to organisms. *J Proteome Res.* **13**, 433-46.

Niehuis O, Judson AK, Gadau J (2008) Cytonuclear genic incompatibilities cause increased mortality in male F2 hybrids of Nasonia giraulti and N. vitripennis. *Genetics* **178**, 413-26.

Nishimura Y, Shikanai T, Kawamoto S, Toh-E A (2020) Step-wise elimination of α-mitochondrial nucleoids and mitochondrial structure as a basis for the strict uniparental inheritance in Cryptococcus neoformans. *Sci Rep*. **10**, 2468.

Mairbäurl H (2013) Red blood cells in sports: effects of exercise and training on oxygen supply by red blood cells. *Front Physiol* **4**, 332

Maklakov AA, Friberg U, Dowling DK, Arnqvist G (2006) Within-population variation in cytoplasmic genes affects female life span and aging in Drosophila melanogaster. *Evolution* **60**, 2081-2086.

Martin WF, Müller M (1998) The hydrogen hypothesis for the first eukaryote. *Nature* **392**, 37–41.

Martin WF, Garg S, Zimorski V (2015) Endosymbiotic theories for eukaryote origin. Philos Trans R Soc Lond B Biol Sci **370**, 20140330.

Mastrantonio V, Latrofa MS, Porretta D, Lia RP, Parisi A, Latta R, Dantas-Torres F, Otranto D, Urbanelli S (2019a) Paternal leakage and mtDNA heteroplasmy in *Rhipicephalus* spp. Ticks. *Scientific Reports* **9**, 1460.

Mastrantonio V, Urbanelli S, Porretta D (2019b) Ancient hybridisation and mtDNA introgression behind current paternal leakage and heteroplasmy in hybrid zones. *Scientific Reports* **9**, 19177.

Meiklejohn CD, Montooth KL, Rand DM (2007) Positive and negative selection on the mitochondrial genome. Trends Genet. **23**, 259-263.

Meiklejohn CD, Holmbeck MA, Siddiq MA, Abt DN, Rand DM, Montooth KL (2013) An Incompatibility between a mitochondrial tRNA and its nuclear-encoded tRNA synthetase compromises development and fitness in Drosophila. *PLoS Genet*. **9**, e1003238.

Metcalfe NB, Van Leeuwen TE, Killen SS (2015) Does individual variation in metabolic phenotype predict fish behaviour and performance? *J Fish Biol*. **88**, 298-321.

Mitchell P (1961) Coupling of Phosphorylation to Electron and Hydrogen Transfer by a Chemi-Osmotic type of Mechanism. *Nature* **191**, 144–148.

Montooth KL, Meiklejohn CD, Abt DN, Rand DM (2010) Mitochondrial-nuclear epistasis affects fitness within species but does not contribute to fixed incompatibilities between species of Drosophila. *Evolution* **64**, 3364-79.

Morales HE, Pavlova A, Joseph L, Sunnucks P (2015) Positive and purifying selection in mitochondrial genomes of a bird with mitonuclear discordance. *Mol Ecol.* **24**, 2820-37.

Morgan JAT, Macbeth M, Broderick D, Whatmore P, Street R, Welch DJ, Ovenden JR (2013) Hybridisation, paternal leakage and mitochondrial DNA linearisation in three anomalous fish (Scombridae). *Mitochondrion* **13**, 852-861.

Morrow EH, Reinhardt K, Wolff JN, Dowling DK (2015) Risks inherent to mitochondrial replacement. *EMBO Reports* **16**, 541-544.

Mossman JA, Biancani LM, Zhu CT, Rand DM (2016a) Mitonuclear epistasis for development time and its modification by diet in Drosophila. *Genetics* **203**, 463–484.

Mossman JA, Tross JG, Li N, Wu Z, Rand DM (2016b) Mitochondrial-nuclear interactions mediate sex-specific transcriptional profiles in *Drosophila*. *Genetics* **204** 613–630.

Mulkidjanian A, Makarova K, Galperin M, Koonin EV (2007) Inventing the dynamo machine: the evolution of the F-type and V-type ATPases. *Nature Reviews Microbiology* **5**, 892–899.

Osada N, Akashi H (2012) Mitochondrial–nuclear interactions and accelerated compensatory evolution: evidence from the primate cytochrome c oxidase complex. *Mol Biol Evol.* **29**, 337–346.

Packert M, Giacalone G, Valvo ML, Kehlmaier C (2019) Mitochondrial heteroplasmy in an avian hybrid form (*Passer italia*: Aves, Passeriformes). *Mitochondrial DNA Part B* **4**, 3809-3812.

Pearl SA, Welch ME, McCauley DE (2009) Mitochondrial heteroplasmy and paternal leakage in natural populations of Silene vulgaris, a gynodioecious plant. *Molecular Biology and Evolution* **26**, 537-545.

Pichaud N, Ballard JW, Tanguay RM, Blier PU (2012) Naturally occurring mitochondrial DNA haplotypes exhibit metabolic differences: insight into functional properties of mitochondria. *Evolution* **66**, 3189-3197.

Pichaud N, Ballard JW, Tanguay RM, Blier PU (2013) Mitochondrial haplotype divergences affect specific temperature sensitivity of mitochondrial respiration. *J Bioenerg Biomembr*. **45**, 25-35.

Pichaud N, Bérubé R, Côté G, Belzile C, Dufresne F, Morrow G, Tanguay RM, Rand DM, Blier PU (2019) Age Dependent Dysfunction of Mitochondrial and ROS Metabolism Induced by Mitonuclear Mismatch. *Front Genet.* **10**, 130

Radojicic JM, Krizmanic I, Kasapidis P, Zouros E (2015) Extensive mitochondrial heteroplasmy in hybrid water frog (*Pelophylax* spp.) populations from Southeast Europe. *Ecology and Evolution* **5**, 4529–4541.

Radzvilavicius AL, Kokko H, Christie J (2017) Mitigating mitochondrial genome erosion without recombination. *Genetics* **207**, 1079-1088

Radzvilavicius A (2021) Beyond the "selfish mitochondrion" theory of uniparental inheritance: A unified theory based on mutational variance redistribution. *Bioessays* **43**, e2100009.

Ramesh MA, Malik SB, Logsdon JM Jr (2005) A phylogenomic inventory of meiotic genes; evidence for sex in Giardia and an early eukaryotic origin of meiosis. *Curr Biol* **15**, 185-91.

Rand DM, Clark AG, Kann L (2001) Sexually antagonistic cytonuclear fitness interactions in *Drosophila melanogaster*. *Genetics* **159**, 173–187.

Rand DM, Haney RA, Fry AJ (2004) Cytonclear coevolution: the genomics of cooperation. *Trends in Ecology and Evolution* **19**, 645-653.

Rand DM, Fry A, Sheldahl L (2006) Nuclear-mitochondrial epistasis and drosophila aging: introgression of Drosophila simulans mtDNA modifies longevity in *D. melanogaster* nuclear backgrounds. *Genetics* **172**, 329-41.

Rank NE, Mardulyn P, Heidl SJ, Roberts KT, Zavala NA, Smiley JT, Dahlhoff EP (2020) Mitonuclear mismatch alters performance and reproductive success in naturally-introgressed populations of a montane leaf beetle. *Evolution* **74**, 1724-1740.

Riesenberg L, Soltis D (1991) Phylogenetic consequences of cytoplasmic gene flow in plants. *Evol. Trend Plants* **5**, 65–84.

Ruiz-Pesini E, Mishmar D, Brandon M, Procaccio V, Wallace DC (2004) Effects of purifying and adaptive selection on regional variation in human mtDNA. Science **303**, 223-226.

Ryan MT, Hoogenraad NJ (2007) Mitochondrial-nuclear communications. Annual Reviews Biochemistry **76**, 701-722.

Sackton TB, Haney RA, Rand DM (2003) Cytonuclear coadaptation in Drosophila: disruption of cytochrome c oxidase activity in backcross genotypes. *Evolution* **57**, 2315-25

Sagan L (1967) On the origin of mitosing cells. *Journal of Theoretical Biology* **14**, 225–274

Salin K, Luquet E, Rey B, Roussel D, Voituron Y (2012) Alteration of mitochondrial efficiency affects oxidative balance, development and growth in frog (*Rana temporaria*) tadpoles. *J Exp Biol* **215**, 863-9.

Salin K, Villasevil EM, Anderson GJ, Lamarre SG, Melanson CA, McCarthy I, Selman C, Metcalfe NB (2019) Differences in mitochondrial efficiency explain individual variation in growth performance. *Proc R Soc B* **286**, 20191466

Sloan DB, Havird JC, Sharbrough J (2017) The on-again, off-again relationship between mitochondrial genomes and species boundaries. *Mol Ecol.* **26**, 2212-2236.

Speijer D, Lukeš J, Eliáš M (2015) Sex is a ubiquitous, ancient, and inherent attribute of eukaryotic life. *Proc Natl Acad Sci USA* **112**, 8827-34.

Speijer D (2016) What can we infer about the origin of sex in early eukaryotes? *Phil. Trans. R. Soc. B* **371,** 20150530

Spirek M, Polakova S, Jatzova K, Sulo P (2014) Post-zygotic sterility and cytonuclear compatibility limits in S. cerevisiae xenomitochondrial cybrids. *Front Genet*. **5**, 454.

Sujkowski A, Spierer AN, Rajagopalan T, Bazzell B, Safdar M, Imsirovic D, Arking R, Rand DM, Wessells R (2019) Mito-nuclear interactions modify Drosophila exercise performance. *Mitochondrion* **47**, 188-205

Sutovsky P, Moreno RD, Ramalho-Santos J, Dominko T, Simerly C, Schatten G (2000) Ubiquitinated sperm mitochondria, selective proteolysis, and the regulation of mitochondrial inheritance in mammalian embryos. *Biol Reprod*. **63**, 582-90.

Taanman JW (1999) The mitochondrial genome: structure, transcription, translation and replication. Biochim Biophys Acta. **1410**, 103-123.

Toews DP, Brelsford A (2012) The biogeography of mitochondrial and nuclear discordance in animals. *Mol Ecol.* **21**, 3907-30.

Trivers RL (1972) Parental investment and sexual selection. In B. Campbell (Ed.), *Sexual selection and the descent of man* (pp. 136-179).

Uetz GW, Roberts JA (2002) Multisensory cues and multimodal communication in spiders: insights from video/audio playback studies. *Brain Behav Evol.* **59**, 222-30.

Wallace DC, Brown MD, Lott MT (1999) Mitochondrial DNA variation in human evolution and disease. *Gene* **238**, 211-230.

Weiss MC, Sousa FL, Mrnjavac N, Neukirchen S, Roettger M, Nelson-Sathi S, Martin WF (2016) The physiology and habitat of the last universal common ancestor. *Nature Microbiology* **1**, 16116

Welch ME, Darnell MZ, McCauley DE (2006) Variable Populations within variable populations: quantifying mitochondrial heteroplasmy in natural populations of the gynodioecious plant *Silene vulgaris. Genetics* **174**, 829–837.

Wolff JN, Pichaud N, Camus MF, Côté G, Blier PU, Dowling DK (2016) Evolutionary implications of mitochondrial genetic variation: mitochondrial genetic effects on OXPHOS respiration and mitochondrial quantity change with age and sex in fruit flies. *J Evol Biol*. **29**, 736-47.

Wolstenholme DR (1992) Animal mitochondrial DNA: structure and evolution. Int Rev Cytol. **141**, 173-216.

Zahavi A (1975) Mate selection-a selection for a handicap. *J Theor Biol* **53**, 205-14.

CHAPTER II | Selection for biparental inheritance of mitochondria under hybridisation and mitonuclear fitness interactions

Published in Proceedings of the Royal Society B, 2021

Tom M Allison[1*], Arunas L Radzvilavicius[2], Damian K Dowling[1]

1. School of Biological Sciences, Monash University, Victoria, Australia

2. Department of Mathematics, University of Bergen, Norway

2.1 | ABSTRACT

Uniparental inheritance of mitochondria predominates over biparental inheritance in most eukaryotes. However, examples of biparental inheritance of mitochondria (BPI), or paternal leakage, are becoming increasingly prevalent. Most reported cases of BPI occur in hybrids of distantly related sub-populations. It is thought that BPI in these cases is maladaptive; caused by a failure of female or zygotic autophagy machinery to recognise divergent male mitochondrial DNA 'tags'. Yet, recent theory has put forward examples in which BPI can evolve under adaptive selection, and empirical studies across numerous metazoan taxa have demonstrated outbreeding depression in hybrids attributable to disruption of population-specific mitochondrial and nuclear genotypes ('mitonuclear mismatch'). Based on these developments, we hypothesise that BPI may be favoured by selection in hybridising populations when fitness is shaped by mitonuclear interactions. We test this idea using a deterministic, simulation-based population genetic model, and demonstrate that biparental inheritance is favoured over strict uniparental inheritance under moderate levels of gene flow typical of hybridising populations. Our model suggests that BPI may be stable, rather than a transient phenomenon, in hybridising populations.

2.2 | INTRODUCTION

It is commonly held that mitochondrial DNA (mtDNA) is inherited strictly down the maternal line in most eukaryotes. From a theoretical perspective, the benefits of uniparental inheritance (UPI) of mtDNA have become increasingly clear. In modern eukaryotes, UPI is thought to supress the spread of 'selfish' mitochondrial mutants (Cosmides & Tooby 1981; Hoekstra 1990), facilitate rapid elimination of deleterious haplotypes and fixation of beneficial haplotypes (Christie & Beekman 2017), and combat mutational erosion in the mitochondrial genome (Radzvilavicius *et al*. 2017). Despite these benefits, evidence has been growing that exceptions to uniparental inheritance are widespread across the eukaryotic domain. With increasing sensitivity and deployment of deep-sequencing technologies, the last two decades have seen a marked increase in the detection of 'paternal leakage' – the inheritance of small quantities of paternal mtDNA – in animal, plant and fungal species (Kondo *et al*. 1990; Schwartz & Vissing 2002; Kvist *et al*. 2003; Fontaine *et al*. 2007; Morgan *et al*. 2013; Nunes *et al*. 2013; Wolff *et al*. 2013; Radojicic *et al*. 2015; Gandolfi *et al*. 2017; Mastrantonio *et al*. 2019a; Mastrantonio *et al*. 2019b; Polovina *et al*. 2020). Paternal leakage can generate a state of heteroplasmy, where multiple divergent mtDNA haplotypes exist within the same cell or individual (Dowling 2014). While the diminutive size of sperm relative to ova strongly biases the ratio of paternal and maternal mtDNA in offspring of many plants and animals, cases of high level intra-individual heteroplasmy (whereby two haplotypes are maintained at appreciable frequencies within an individual) have been increasingly reported (Solignac *et al*. 1983; Van Leeuwen *et al*. 2008; Magnacca & Brown 2010; Woloszynska 2010; Robison *et al*. 2015; Kastally & Mardulyn 2017).

Currently, the factors underlying the observed patterns of paternal leakage remain obscure, though most reported cases occur among hybrids of distantly related populations and subspecies (Wilson & Xu 2012; MacCauley 2013; Ladoukakis & Zouros 2017). Such cases of paternal leakage have classically been attributed to a failure of female oocytes to recognise and subsequently eliminate 'foreign' male mtDNA (Ladoukakis & Zouros 2017, Sato & Sato 2017). Our current mechanistic understanding is that males attach a molecular 'tag' to sperm mitochondria which can then be recognized by female or zygotic autophagy machinery after fertilization (Sato & Sato 2017). If, however, there is little interbreeding between subpopulations, over time molecular markers will likely diverge and the molecular tag used by males in one subpopulation may become unrecognizable to the female mitochondrial destruction machinery in the other. Under this scenario, there is a breakdown in the intersexual interplay maintaining uniparental inheritance, and biparental inheritance (BPI) may occur in hybrids. Despite a growing understanding of the mechanisms underpinning BPI in hybrids, currently we lack clear predictions as to whether we should expect these instances of paternal leakage to be quickly selected against and thereby transient, or beneficial and thus maintained by selection.

Recent theory has identified scenarios in which biparental inheritance may persist adaptively within species; either as a result of sexual conflict over primary control of mitochondrial inheritance (with male control favouring some paternal leakage), or because paternal leakage may mitigate the hypothesised accumulation of male-harming mtDNA variants expected under strictly maternal inheritance (Radvilavicius *et al*. 2017; Kuijper *et al*. 2015). While insightful, this previous work focussed on situations where there is variation in mtDNA but no variation in the mitochondrial-associated nuclear genotype (i.e. among

nuclear genes with mitochondrial function) and consequently it fails to capture the dynamics in hybridising populations where variation in both genomes may exist. Thus, we lack a clear understanding for why biparental inheritance frequently persists in sympatric populations of related taxa despite potentially considerable interpopulation gene flow (Kvist *et al*. 2003; Welch *et al*. 2006; Pearl *et al*. 2009; Morgan *et al*. 2013; Radojicic *et al*. 2015; Mastrantonio *et al*. 2019a; Mastrantonio *et al*. 2019b; Packert *et al*. 2019). While these empirical observations suggest that recurrent hybridisation may itself select for biparental inheritance of mitochondria, such a contention demands rigorous assessment, particularly given the suite of theoretical benefits offered by uniparental inheritance of mitochondria.

In order to capture the evolutionary dynamics of segregating variation in both nuclear and mitochondrial genomes, it is important to consider that organismal fitness is contingent upon the interaction between the two genomes. Evidence suggests that tight coordination between proteins from the two genomes is required for precise function of the electron transport system, as well as mitochondrial transcription, translation and other regulatory processes (Rand *et al*. 2004; Lane 2005; Woodson & Chory 2008; Wolff *et al*. 2014), and previous work has confirmed strong signatures of molecular coevolution between mitochondrial and nuclear genomes (Blier *et al*. 2001; Rand *et al*. 2004; Osada & Akashi 2012; Barreto *et al*. 2018). It is perhaps unsurprising, then, to find that disruption of putatively coevolved combinations (either naturally under hybridisation or experimentally using 'cybrid' individuals) has on numerous occasions been associated with poor bioenergetic and phenotypic function (Barrientos *et al*. 1998; Dey *et al*. 2000; Sackton *et al*. 2003; McKenzie *et al*. 2004; Ellison & Burton 2006; Meiklejohn *et al*. 2013; Chang *et al*. 2016; Ma *et al*. 2016; Dobler *et al*. 2018; Pichaud *et al*. 2019; Rank *et al*. 2020). Indeed,

'mitonuclear mismatches' (the union of putatively novel mitochondrial and nuclear genotypes that lack a recent coevolutionary history) in hybrids have frequently been proposed as ubiquitous drivers of outbreeding depression and reproductive isolation (Dowling *et al*. 2008; Gershoni *et al*. 2009; Burton & Barreto 2012; Hill 2019).

In the present paper, we test the hypothesis that the fitness burden of mitonuclear mismatches in hybridising populations may be alleviated by allowing biparental inheritance of mitochondria. Accordingly, we explore whether biparental inheritance may be selected for under certain rates of hybridisation, thus helping to explain previous empirical reports of persistent paternal leakage in sympatric hybridising populations. Thus, we define a hypothetical 'mitochondrial inheritance mode' allele that encodes biparental inheritance when distantly related individuals mate and uniparental inheritance when closely related individuals mate. We term this allele 'mate-specific biparental inheritance', and herein provide a mathematical model competing strict UPI against mate-specific BPI, to assess whether such an allele would be favoured or eliminated in hybridising populations, under an assumption of a mitonuclear interactive effect on fitness.

2.3 | THE MODEL

In order to assess the adaptive value of a mate-specific BPI trait in a hybrid zone, we observe a metapopulation of single-celled, diploid organisms comprised of two distinct lineages ('populations' hereafter). The populations are arranged along a one-dimensional cline consisting of 50 'demes': subpopulations wherein individuals mate and between which individuals migrate. We track a single mitochondrial locus with two alleles – 0 and 1 – and

an interacting nuclear locus – also with two alleles ***A*** and ***a*** – such that each population is characterised by a particular co-evolved combination (***A***/0 and ***a***/1 respectively).

We also track a second nuclear locus that controls the inheritance mode of mitochondria. One allele (*U*) codes for strict uniparental inheritance, whereas the other (*u*) codes for mate-specific biparental inheritance. This allele is completely linked with a sex-determination locus, such that it is only expressed in females at the gamete stage, as seen in many unicellular eukaryotes (Fedler *et al*. 2009; Shakya & Idnurm 2014; Speijer *et al*. 2015; Coelho *et al*. 2018; Sun *et al*. 2020).

In the model, female gametes with the *U* allele always reject their partner's mitochondria and the full number of mitochondria in the offspring is restored by replication of existing mitochondria. Female gametes with the *u* allele will accept their partner's mitochondria *if and only if* their N-mt alleles *do not* match (ie. ***A*** meets ***a***). Otherwise, zygote formation will proceed with uniparental inheritance as normal. This is consistent with our current understanding of mitochondrial recognition systems, which depend on expression of nuclear genes for the molecular tag that is attached to sperm (Ladoukakis & Zouros 2017, Sato & Sato 2017).

Each cell contains *M* mitochondria in the diploid (zygote/adult) stage. Fitness is determined by the proportion of each mitochondrial haplotype given a particular nuclear background. It is assessed in the diploid stage and thus its description requires three distinct fitness curves (one for each of the possible N-mt genotypes – ***AA***, ***Aa*** and ***aa***). We model two different sets of fitness curves in order to test two distinct hypotheses for the beneficial effects of a

putative mate-specific BPI allele. Under both model variations, homozygote fitness follows a concave curve, meaning that fitness declines more rapidly with each successive addition of a mismatched mitochondrion. This kind of fitness curve is thought to best explain the observation of 'threshold' effects, where heteroplasmic individuals display mild symptoms up to a critical frequency of 'mutant' mitochondria, after which disease may be severe (Rossignol *et al*. 2003).

The first hypothesis – which we term the 'risk avoidance' hypothesis – is based solely on the idea that biparental inheritance of mitochondria reduces the risk of producing descendants with a complete mitonuclear mismatch. This idea is best tested by attributing a flat cost to all hybrids (ie. there is no fitness benefit for being in a state of heteroplasmy *per se*). Any fitness advantage gained through biparental inheritance and subsequent heteroplasmy in this scenario is necessarily manifested solely in increased fitness of homozygous descendants.

The second hypothesis – which we term the 'heteroplasmic-advantage' hypothesis – proposes that the biparental inheritance of mitochondria brought about by reproduction between individuals of divergent lineages may confer direct fitness gains to hybrids because it ensures that the haploid contribution of N-mt genes transmitted by each parent is paired to at least some matching mitochondria. We see this hypothesis belonging to a broader class of evolutionary models wherein an allele may increase in frequency in a hybrid zone if it improves hybrid fitness (or rather minimises outbreeding depression) and carries a sufficiently small cost to homozygotes of the parent populations (Sanderson 1989). To assess this idea, we explicitly incorporate some additional benefit to a hybrid possessing

both types of mitochondria. Hybrids still suffer a fitness cost relative to homozygotes, but we assume a relative fitness benefit for heterozygotes with intermediate amounts of each mitochondrial haplotype (see figure S1 in Electronic Supplementary Material for fitness curves). In each variation, strength of selection may be modified using a selection coefficient s, which varies between 0 and 1, with larger values representing a more drastic mitonuclear mismatch.

A complete model cycle is characterised by selection (on diploid cells), migration of surviving cells between demes (at rate h), meiosis (with recombination between the N-mt locus and inheritance-mode locus) and finally random mating within demes. For precise description of these processes, see Methods in Supplementary Material.

We begin the simulation with a metapopulation where ***AA*** individuals populate the first 25 demes and ***aa*** individuals populate the remaining demes and allow it to reach migration/selection balance. We proceed by injecting the *u* allele at a frequency of 0.01 across the cline (into each deme). Although it may be more biologically plausible to assume that paternal leakage is the default mode and show that uniparental inheritance does *not* invade such a population, we thought it a stronger display of the adaptive benefits of mate-specific BPI to show this trait invading a population. The ultimate goal remains to show the conditions under which biparental inheritance of mitochondria may be beneficial. The simulation was stopped when *u* reached an equilibrium frequency (defined as a frequency change of $<10^{-10}$ between generations) or after 75,000 generations, whichever came first. We tracked invasion of the mate-specific BPI allele *u* under a wide range of migration (h)

and selection (*s*) values, though we limit migration to $0 < h < 0.25$, such that there are no cases where more than half of the individuals leave the deme in which they were formed.

2.4 | RESULTS

We found that the frequency of the *u* allele (*p*) always converges on a single stable equilibrium $0 \leq p \leq 1$ under the parameter space explored (Figure 1). In the heteroplasmic-advantage model, *u* invades under the majority of migration/selection combinations, whereas in the risk-avoidance model *u* invades in a smaller region of parameter space. In each case, the parameter space where the allele is more likely to invade corresponds to lower selection strength and higher migration, where gene flow is higher. Our results show that *u* is always beneficial to hosts that also possess the uncommon N-mt allele for a given region of the cline (Figure 2A) and that higher gene flow allows this benefit to be realised in more cells (due to increased proportions of the uncommon allele), thus explaining the link between gene flow and invasion of the mate-specific BPI allele. Under the heteroplasmic-advantage model, increasing hybrid fitness can allow for an additional increase in frequency of the uncommon allele in the opposing subpopulation's range, in turn furthering the range of demes where the benefits of *u* can be realised. For this reason, along with the simple additive benefits of increased hybrid fitness when in a state of heteroplasmy, *u* invades over a larger portion of parameter space under the heteroplasmic-advantage model.

2.4.1 | Risk Avoidance model

To investigate the reasons underlying spread or elimination of *u* under the risk-avoidance model, we examine some basic population statistics while keeping migration rate constant and varying selection strength; and vice versa by varying migration rate while keeping the selection strength constant. We also examine the effects of varying *p* in the metapopulation by keeping *u* at a fixed frequency during simulations.

Both migration rate and selection strength strongly influence the distribution of N-mt alleles ***A*** and ***a*** along the cline (Figure S2 – ESM). Increasing selection strength has a similar effect to decreasing migration rate: each reduces gene flow. The effect is a narrower cline and more unequal gene frequencies in the two adjacent demes comprising the cline centre (demes 25 and 26) (Figure $S2_{A,C}$). The two may act in concert to produce particularly high or low gene flow (Figure $S2_{E}$). Examining the frequency of the 'majority' N-mt allele in the cline centre over multiple fixed frequencies of *u*, we see that that while migration and selection strongly determine the distribution of genotypes, the frequency of *u* plays a comparatively small role (Figure $S2_{B,D,F}$).

Varying *u*, however, strongly affects the distribution of mitochondrial alleles (Figure S3 – ESM). In the cline centre, heteroplasmy increases with increasing *u*, even approaching the theoretical limit where all cells exhibit a 50:50 mix of each haplotype. Since heteroplasmic cells cannot achieve either maximum or minimum possible fitness, increasing the frequency of *u* reduces variance in fitness. Thus, *fitness* of each N-mt genotype across the cline is affected by both migration/selection ratio and frequency of *u*. By comparing cells of the same N-mt genotype and different mitochondrial inheritance modes (for example, ***AA****U* vs

AA*u*[1]), we see that there are some regions in the cline where *u* is beneficial to its host and others where it is detrimental (Figure 2_A).

Homozygotes with strict UPI perform equally well or better than mate-specific BPI homozygotes when on 'their side' of the cline (where the N-mt allele they possess is the most common). This is true for all migration/selection combinations explored. When a particular N-mt allele is overwhelmingly more common, any mate-specific BPI gametes encountering an (albeit rare) opposing N-mt allele will result in heteroplasmy and – given the high probability that this allele finds itself in a homozygote in the next generation – sentence any descendants to a reduction in maximum fitness. Note that for this to happen, there must be at least some probability of a gamete encountering an opposing N-mt allele. This explains why the fitness differences are most noticeable a few demes on either side of the cline centre and taper off to almost zero toward either end of the cline (corresponding to regions of moderate and very low likelihood of encountering a mismatched N-mt allele, respectively).

Conversely, having the mate-specific BPI allele provides an advantage in homozygotes when the opposing N-mt allele is the dominant type (***AA****u* individuals perform better than ***AA****U* individuals in a predominantly ***a*** environment). We can imagine ***a*** allele gametes with strict UPI in a predominantly ***a*** environment (genotype ***a****U*): upon encountering an ***A*** gamete and forming a zygote, subsequent recombination means that half of these encounters will

[1]for statistical representation of the metapopulation in Results we only consider the inheritance mode locus (*U*/*u*) when it is expressed, which only occurs in females. Although both alleles are incorporated into the model for males, for this analysis we average the fitness over both *U* and *u* males. This leaves us with 6 functionally different genotypes to assess, rather than the 16 of a classic two-locus model.

produce a gamete with ***A*** at the N-mt locus, *U* at the inheritance mode locus and all type 1 mitochondria (a completely mitonuclear mismatched gamete). Any subsequent encounter between the now mismatched ***A***/1 gamete and another gamete will at best produce a hybrid with intermediate fitness and at worst (upon encountering another rare ***A*** allele) a mismatched homoplasmic homozygote – the lowest fitness genotype. The benefit of *u*, therefore, is in avoiding this situation. There is very little chance of producing a mismatched homoplasmic zygote since any pairing between an '***a****u*' gamete and an ***A*** gamete will result in heteroplasmy, as demonstrated in the idealised schematic in Figure 3. Thus, while *u* precludes maximum fitness, it also prevents formation of the lowest fitness genotypes. This can be seen in the reduced variance of the *u* genotypes compared to their matched N-mt genotype counterparts (Figure 2_B).

The result of tensions between benefits of *u* when paired with the rarer N-mt allele and the costs of *u* when paired with the common N-mt allele is a net benefit for *u* in the cline centre (demes 25 and 26) and a net cost in the demes on either side (Figure $2_{C,D}$). Note that this is only true for cases where *u* invades, as there is no net benefit of *u* in the cline centre under low gene flow due to a lower rate of interactions between ***A*** and ***a*** alleles. These trends are merely exaggerated by increasing the frequency of *u*. But while the benefit of *u* in the cline centre increases in a roughly linear fashion, the cost of *u* on either side increases quadratically with increasing frequency of the *u* allele (Figure 2_E). Thus, an invasion may begin with some demes overproducing *u* and the allele spreading, but as the magnitude of benefits and costs change under the growing frequency of *u*, elimination of *u* in some regions may catch up to production in others. A stable intermediate frequency is reached when net production in some regions equals the net elimination in others. Due to persistent

net production in the cline centre and net elimination elsewhere, we see a hump shape in the clinal distribution of the *u* allele during invasion and at equilibrium (Figure S4 – ESM).

2.4.2 | Heteroplasmic advantage model

The reasons for spread of *u* under the heteroplasmic advantage model are broadly similar, such that many of the results already discussed also apply to this model. There are, however, two key differences. Firstly, since heterozygotes can achieve higher fitness with heteroplasmy, N-mt genotype frequencies may be more strongly altered by increasing frequency of *u* (Figure S5 – ESM). Essentially, under higher frequencies of *u*, increased hybrid fitness means that the N-mt alleles and their associated mitochondria are able to persist further into the other population's range (the cline is widened). Particularly under moderate gene flow, increasing the frequency of *u* in the metapopulation increases the cline width and produces a more even ratio of each homozygote in the cline centre. This broadens the regions where 'risk-avoidance' advantages may be realised.

Secondly, the heteroplasmic advantage itself contributes greatly toward increasing fitness of those cells possessing the *u* allele. This acts in an additive fashion upon the already established benefits of risk avoidance. This additive benefit is enough to frequently overturn a deme from net elimination to net production of the *u* allele. As a consequence of these two differences acting in conjunction, the migration/selection conditions under which *u* invades is much greater.

2.5 | DISCUSSION

We sought to provide a mathematical test of the hypothesis that biparental inheritance of mitochondria confers an adaptive advantage during episodes of hybridisation between populations. The hypothesis is based on the assumption that when individuals from divergent populations hybridise, interactions between mitochondrial and nuclear gene products may disrupt electron transport system function and mitochondrial regulation systems, causing a loss of phenotypic function (Rand *et al*. 2004; Dowling *et al*. 2008; Barreto *et al*. 2018; Hill *et al*. 2019). Our results show that when divergent populations hybridise under higher rates of gene flow, mate-specific biparental inheritance may benefit populations by avoiding production of completely mitonuclear-mismatched organisms. The implication is that when populations hybridise under certain levels of gene flow, we may expect biparental inheritance of mitochondria to be stable and adaptive, as opposed to maladaptive and rapidly selected against.

We show that the net benefits of a mate-specific BPI allele are always highest in the cline centre, where the likelihood of encountering a gamete from a foreign population is greatest. This implies that the risk of producing a mitonuclear-mismatched cell is underpinned by uncertainty in the origin of potential mating partners. We can imagine that when pre-zygotic barriers to reproduction are yet to develop and mate selection is random, there is a high risk of choosing a partner that leads to a poor mitonuclear match in the contact zone. Though the uncertainty in mate-selection is not affected by possessing the mate-specific BPI allele, the state of heteroplasmy that is associated with this allele in the hybrid zone means that individuals with this genotype have a lower risk of producing descendants with a total mitonuclear mismatch. We show that under weak to moderate selection, a genotype that

sacrifices the highest fitness phenotype in return for avoiding the lowest fitness phenotype will enjoy higher mean fitness and hold an evolutionary advantage.

We show a putative mate-specific BPI allele invading a population of strict UPI cells, however it is important to note that under hybridisation through secondary contact, mate-specific BPI may already be the default state (Ladoukakis & Zouros 2017). Biologically, we refer to cases where molecular tags, which are normally attached to male mitochondrial DNA to signal destruction, have diverged in the intervening period of isolation and biparental inheritance proceeds due to a failure of female recognition systems. Our results suggest that if mate-specific BPI is already present in hybridising populations due to diverged molecular recognition systems - and providing that this divergence is also sufficient to disrupt interactions between mitochondrial and nuclear gene products – then any allele restoring the recognition systems required for strict UPI may facilitate worse mitonuclear matches and be swiftly eliminated. In light of this, a testable prediction coming from our model is that the divergence of mitochondrial recognition and destruction mechanisms should diverge between populations at a faster rate than expected by drift alone.

Mathematically, however, the distinction between a mate-specific BPI allele invading a population of strict UPI individuals and a strict UPI allele invading a population of mate-specific BPI individuals is redundant, as our results show a single stable equilibrium frequency of u throughout the cline (in other words, the starting frequency of mate-specific BPI alleles does not affect the equilibrium frequency). This means that our results offer new predictions for the occurrence of biparental inheritance in clines where hybridisation is the result of environmental gradients rather than secondary contact. Our current understanding

of how mitochondrial molecular recognition systems fail does not address situations where mitochondrial divergence has occurred despite persistent gene flow between hybridising populations; as may be the case under spatially driven local adaptation. Under this type of hybridisation, we may not *a priori* expect that male-mitochondrial recognition systems would diverge at all. If, however, an environmental gradient supports a mitochondrial polymorphism and there is subsequent compensatory nuclear evolution, our model predicts that we should in fact expect to see evolution away from strict uniparental inheritance toward mate-specific biparental inheritance (provided there is enough migration between the two sub-populations). This is a particularly interesting prediction, as it suggests that paternal leakage could be driven by the requirement for sustaining mitochondrial function, rather than by allopatric divergence alone. We suggest that further empirical investigations into rates of heteroplasmy in hybrid zones along environmental gradients known to shape mitochondrial evolution, such as temperature and altitude (Cheviron & Brumfield 2009; Balloux *et al*. 2009; Camus *et al*. 2017), will be insightful.

We also demonstrate that if being in a state of heteroplasmy reduces outbreeding depression, biparental inheritance will be beneficial over a wide range of migration and selection conditions. The requisite assumption may at first seem extreme, since some empirical findings and theoretical models suggest that heteroplasmy might reduce fitness (Sharpley *et al*. 2012; Christie *et al* 2015). We note, however, that empirical studies have only been carried out on nuclear-homozygous organisms and that there is a lack of empirical evidence comparing fitness consequences of homoplasmy and heteroplasmy in hybrids. We suggest here that if both nuclear alleles are expressed then each mitochondrion could potentially receive the correctly matched nuclear-encoded mitochondrial products. Indeed,

it is thought that mitochondria-specific molecular recognition sites aid in targeting nuclear encoded products to the required organelle (Glover & Lindsay 1992; Lane 2005; Williams *et al*. 2014), raising the intriguing prospect that a haplotype could 'request' products specifically from its matched nuclear allele.

As an alternative explanation, in the case of metazoans with multiple tissues, possessing two distinct mitochondrial haplotypes in conjunction with their respective coevolved nuclear alleles in a single individual may provide more variation upon which tissue-specific selection of mitochondria can act. The idea of differential selection on components of mitochondrial function across tissues remains an open question (Wolff *et al*. 2014), though there are some examples (Jenuth *et al*. 1997; Fan *et al*. 2008; Stewart *et al*. 2008; Ma *et al*. 2014). Given that mitochondrial proteomes differ greatly between tissues (Mootha *et al*. 2003), differential selection for mitochondrial function leaves open the possibility of tissue-specific segregation of mitochondrial haplotypes. In this case, having two sets of mitochondrial haplotypes would always be *at least* as good as one set (if one set performed best in all tissues) and perhaps better if different sets functioned best in different tissues. The net result, in the latter case, would be a higher mean fitness across all tissues and a likely higher individual fitness. We suggest that further empirical studies into tissue-specific selection of mitochondria based on function will likely provide greater insight into this hypothesis.

While the heteroplasmic-advantage model remains more speculative than the risk-avoidance model, it still provides an interesting example of how a trait reducing outbreeding depression can be selected for in a hybrid zone, even if it carries costs to

homozygotes of the parent populations. Previous modelling work has demonstrated that such a trait can be selected for within the central hybrid zone without increasing in frequency at all in the rest of the cline (Sanderson 1989). Our study offers new insights into this under-explored side of hybridisation (Coyne & Orr 2004). While previous work has modelled fixed costs for homozygotes of parent populations, we show that allowing variation in the fitness of homozygotes throughout the cline (in our case depending on the load of each mitochondrial haplotype and in turn the frequency of the invading *u* allele) can in some cases allow spread of the invading allele throughout the entire population. In other words, the mate-specific BPI trait we present here is a trait that reduces outbreeding depression, carries costs for homozygotes of the parent populations, but is not necessarily a 'rare allele' as classically presented (Barton & Hewitt 1985; Sanderson 1989; Schilthuizen *et al*. 2001). Thus, our results provide support for the adaptive value of alleles reducing outbreeding depression without providing support for the rare-allele phenomenon – suggesting that these 'rare' alleles may be even more common than empirical findings currently suggest.

ACKNOWLEDGEMENTS

We are grateful to Tim Connallon, Sean Layh, Craig White and Luke McKenzie-McHarg, for thoughtful discussions and mathematical insights. This research was supported through the Australian Research Council's Discovery Projects funding scheme (project DP210102931). T.A. was supported by a scholarship from the Australian Government Research Training Program.

2.6 | REFERENCES

Balloux F, Lawson Handley LJ, Jombart T, Liu H, Manica A (2009) Climate shaped the worldwide distribution of human mitochondrial DNA sequence variation. *Proceedings of the Royal Society B: Biological Sciences* **276**, 3447-3455.

Barreto FS, Watson ET, Lima TG, Willett CS, Edmands S, Li W, Burton RS (2018) Genomic signatures of mitonuclear coevolution across populations of *Tigriopus californicus*. *Nature Ecology & Evolution* **2**, 1250-1257.

Barrientos A, Kenyon L, Moraes CT (1998) Human xenomitochondrial cybrids. Cellular models of mitochondrial complex I deficiency. *Journal of Biological Chemistry* **273**, 14210-14217.

Blier PU, Dufresne F, Burton RS (2001) Natural selection and the evolution of mtDNA-encoded peptides: evidence for co-adaptation. *TRENDS in Genetics* **17**, 400-406.

Barton NH, Hewitt GM (1985) Analysis of Hybrid Zones. *Annual Review of Ecology and Systematics* **16**, 113-148.

Burton RS, Barreto FS (2012) A disproportionate role for mtDNA in Dobzhansky-Muller incompatibilities? *Molecular Ecology* **21**, 4942-4957.

Camus MF, Wolff JN, Sgro CM, Dowling DK (2017) Experimental support that natural selection has shaped the latitudinal distribution of mitochondrial haplotypes in Australian *Drosophila melanogaster* **34**, 2600-2612.

Chang CC, Rodriguez J, Ross J (2016) Mitochondrial-nuclear epistasis impacts fitness and mitochondrial physiology of interpopulation *Caenorhabditis briggsae* hybrids. *G3 (Bethesda)* **6**, 209-219.

Cheviron ZA, Brumfield RT (2009) Migration-selection balance and local adaptation of mitochondrial haplotypes in Rufous-collared sparrows (*Zonotrichia capensis*) along an elevational gradient. *Evolution* **63**, 1593-1605.

Christie JR, Beekman M (2017) Uniparental inheritance promotes adaptive evolution in cytoplasmic genomes. *Molecular Biology and Evolution* **34**, 677-691.

Coelho SM, Gueno J, Lipinska AP, Cock JM, Umen JG (2018) UV chromosomes and haploid sexual systems. *Trends in Plant Sciences* **23**, 794-807.

Cosmides L, Tooby J (1981) Cytoplasmic inheritance and intragenomic conflict. *Journal of Theoretical Biology* **89**, 83-129.

Coyne JA, Orr HA (2004) *Speciation*. Oxford University Press, Oxford, UK

Dey R, Barrientos A, Moraes CT (2000) Functional constraints of nuclear-mitochondrial DNA interactions in xenomitochondrial rodent cell lines. *Journal of Biological Chemistry* **275**, 31520-31527.

Dobler R, Dowling DK, Morrow EH, Reinhardt K (2018) A systematic review and meta-analysis reveals pervasive effects of germline mitochondrial replacement on components of health. *Human Reproduction Update* **24**, 519-534.

Dowling DK, Friberg U, Lindell J (2008) Evolutionary implications of non-neutral mitochondrial genetic variation. *Trends in Ecology & Evolution* **23**, 546-554.

Dowling DK (2014) Evolutionary perspectives on the links between mitochondrial genotype and disease phenotype. *Biochemica Et Biophysica Acta-General Subjects* **1840**, 1393-1403.

Ellison CK, Burton RS (2006) Disruption of mitochondrial function in interpopulation hybrids of Tigriopus californicus. *Evolution* **60**, 1382-1391.

Fan W, Waymire KG, Narula N, Li P, Rocher C, Coskun PE, Vannan MA, Narula J, Macgregor GR, Wallace DC (2008) A mouse model of mitochondrial disease reveals germline selection against severe mtDNA mutations. *Science* **319**, 958-962.

Fontaine KM, Cooley JR, Simon C (2007) Evidence for paternal leakage in hybrid periodical cicadas (Hemiptera: *Magicicada* spp.). *PLOS ONE* **2**: e892

Gandolfi A, Crestanello B, Fagotti A, Simoncelli F, Chiesa S, Girardi M, Giovagnoli E, Marangoni C, Di Rosa I, Lucentini L (2017) New evidences of mitochondrial DNA heteroplasmy by putative paternal leakage between the Rock Partridge (*Alectoris graeca*) and the Chukar Partridge (*Alectoris chukar*). *Public Library of Sciences ONE* **12**: e0170507

Gershoni M, Templeton AR, Mishmar D (2009) Mitochondrial bioenergetics as a major motive force of speciation. *BioEssays* **31**, 642-650.

Glover LA, Lindsay JG (1992) Targeting proteins to mitochondria: a current overview. *Biochemical Journal* **284**, 609-620.

Hadjivasiliou Z, Pomiankowski A, Seymour RM, Lane N (2012) Selection for mitonuclear co-adaptation could favour the evolution of two sexes. *Proceedings of the Royal Society B: Biological Sciences* **279**, 1865-1872.

Hill GE, Havird JC, Sloan DB, Burton RS, Greening C, Dowling DK (2019) Assessing the fitness consequences of mitonuclear interactions in natural populations. *Biological Reviews* **94**, 1089-1104

Hoekstra RF (1990) Evolution of uniparental inheritance of cytoplasmic DNA. In Maynard-Smith J and Vida J (eds.) *Organisational Constraints on the Dynamics of Evolution*. Manchester University Press, Manchester, UK

Kastally C, Mardulyn P (2017) Widespread co-occurrence of two distantly related mitochondrial genomes in individuals of the leaf beetle *Gonioctena intermedia*. *Biology Letters* **13**, 20170570

Kondo R, Satta Y, Matsuura ET, Ishiwa H, Takahata N, Chigusa SI (1990) Incomplete maternal transmission of mitochondrial DNA in Drosophila. *Genetics* **126**, 657-663.

Kvist L, Martens J, Nazarenko AA, Markku O (2003) Paternal leakage of mitochondrial DNA in the Great Tit (*Parus major*). *Molecular Biology and Evolution* **20**, 243-247.

Ladoukakis ED, Zouros E (2017) Evolution and inheritance of animal mitochondrial DNA: rules and exeptions. *Journal of Biological Research-Thessaloniki* **24**: 2

Lane N (2005) *Power, sex, suicide: Mitochondria and the meaning of life*. Oxford University Press, New York, USA.

Nunes MDS, Dolezal M, Schlotterer C (2013) Extensive paternal mtDNA leakage in natural populations of *Drosophila melanogaster*. *Molecular Ecology* **22**, 2106-2117.

Ma H, Marti Gutierrez N, Morey R, Van Dyken C, Kang E, Hayama T, Lee Y, Li Y, Tippner-Hedges R, Wolf DP, Laurent LC, Mitalipov S (2016) Incompatibility between nuclear and mitochondrial genomes contributes to an interspecies reproductive barrier. *Cell Metabolism* **24**, 283-294.

Ma H, Xu H, O'Farrell (2014) Transmission of mitochondrial mutations and action of purifying selection in *Drosophila*. *Nature Genetics* **46**, 393-397.

Magnacca KN, Brown MJF (2010) Mitochondrial heteroplasmy and DNA barcoding in Hawaiian *Hylaeus* (*Nesoprosopis*) bees (Hymenoptera: Colletidae). *BMC Ecology and Evolution* **10**: 1742010

Mastrantonio V, Latrofa MS, Porretta D, Lia RP, Parisi A, Latta R, Dantas-Torres F, Otranto D, Urbanelli S (2019a) Paternal leakage and mtDNA heteroplasmy in *Rhipicephalus* spp. Ticks. *Scientific Reports* **9**, 1460.

Mastrantonio V, Urbanelli S, Porretta D (2019b) Ancient hybridisation and mtDNA introgression behind current paternal leakage and heteroplasmy in hybrid zones. *Scientific Reports* **9**, 19177.

McCauley DE (2013) Paternal leakage, heteroplasmy, and the evolution of plant mitochondrial genomes. *New Phytologist* **200**, 966-977.

McKenzie M, Trounce IA, Cassar CA, Pinkert CA (2004) Production of homoplasmic xenomitochondrial mice. *Proceedings of the National Academy of Sciences of the United States of America* **101**, 1685-1690.

Meiklejohn CD, Holmbeck MA, Siddiq MA, Abt DN, Rand DM, Montooth KL (2013) An incompatibility between mitochondrial tRNA and its nuclear-encoded tRNA synthetase compromises development and fitness in *Drosophila*. *PLOS Genetics* **9**: e1003238.

Mootha VK, Bunkenborg J, Olsen JV, Hjerrild M, Wisniewski JR, Stahl E, Bolouri MS, Ray HN, Sihag S, Kamal M, Patterson N, Lander ES, Mann M (2003) Integrated analysis of protein composition, tissue diversity, and gene regulation in mouse mitochondria. *Cell* **115**, 629-640.

Morgan JAT, Macbeth M, Broderick D, Whatmore P, Street R, Welch DJ, Ovenden JR (2013) Hybridisation, paternal leakage and mitochondrial DNA linearisation in three anomalous fish (Scombridae). *Mitochondrion* **13**, 852-861.

Osada N, Akashi H (2012) Mitochondrial-nuclear interactions and accelerated compensatory evolution: evidence from the primate cytochrome C oxidase complex. *Molecular Biology and Evolution* **29**, 337-346.

Packert M, Giacalone G, Valvo ML, Kehlmaier C (2019) Mitochondrial heteroplasmy in an avian hybrid form (*Passer italia*: Aves, Passeriformes). *Mitochondrial DNA Part B* **4**, 3809-3812.

Pearl SA, Welch ME, McCauley DE (2009) Mitochondrial heteroplasmy and paternal leakage in natural populations of Silene vulgaris, a gynodioecious plant. *Molecular Biology and Evolution* **26**, 537-545.

Pichaud N, Berube R, Cote G, Belzile C, Dufresne F, Morrow G, Tanguay RM, Rand DM, Blier PU (2019) Age dependent dysfunction of mitochondrial and ROS metabolism induced by mitonuclear mismatch. *Frontiers in Genetics* DOI: 10.3389/fgene.2019.00130.

Polovina ES, Parakatselaki ME, Ladoukakis ED (2020) Paternal leakage of mitochondrial DNA and maternal inheritance of heteroplasmy in Drosophila hybrids. *Scientific Reports* **10**: 2599.

Radojicic JM, Krizmanic I, Kasapidis P, Zouros E (2015) Extensive mitochondrial heteroplasmy in hybrid water frog (*Pelophylax* spp.) populations from Southeast Europe. *Ecology and Evolution* **5**, 4529–4541.

Radzvilavicius AL, Kokko H, Christie J (2017) Mitigating mitochondrial genome erosion without recombination. *Genetics* **207**, 1079-1088.

Radzvilavicius AL, Hadjivasiliou Z, Pomiankowski A, Lane N (2016) Selection for mitochondrial quality drives evolution of the germline. *PLoS Biology* **14**, e2000410

Rand DM, Haney RA, Fry AJ (2004) Cytonuclear coevolution: the genomic of cooperation. *Trends in Ecology & Evolution* **19**, 645-653.

Rank NE, Mardulyn P, Heidl SJ, Roberts KT, Zavala NA, Smiley JT, Dahlhoff EP (2020) Mitonuclear mismatch alters performance and reproductive success in naturally introgressed populations of a montane leaf beetle. *Evolution* **74**, 1724-1740.

Robison GA, Balvin O, Schal C, Vargo EL, Booth W (2015) Extensive mitochondrial heteroplasmy in natural populations of a resurging human pest, the bed bug (Hemiptera: Cimicidae). *Journal of Medical Entomology* **52**, 734-738.

Rossignol R, Faustin B, Rocher C, Malgat M, Mazat JP, Letellier T (2003) Mitochondrial threshold effects. *Biochemical Journal* **15**, 751-762.

Sackton TB, Haney RA, Rand DM (2003) Cytonuclear coadaptation in Drosophila: disruption of cytochrome c oxidase activity in backcross genotypes. *Evolution* **57**, 2315-2325.

Sanderson N (1989) Can gene flow prevent reinforcement? *Evolution* **43**, 1223-1235.

Sato K, Sato M (2017) Multiple ways to prevent transmission of paternal mitochondrial DNA for maternal inheritance in animals. *Journal of Biochemistry* **162**, 247-253.

Schilthuizen M, Hoekstra RF, Gittenburger E (2001) The 'rare allele phenomenon' in a ribosomal spacer. *Molecular Ecology* **10**, 1341-1345

Schwartz M, Vissing J (2002) Paternal inheritance of mitochondrial DNA. *New England Journal of Medicine* **347**, 576-580.

Shakya VPS, Idnurm A (2014) Sex determination directs uniparental mitochondrial inheritance in Phycomyces. *Eukaryotic Cell* **13**, 186-189.

Sharpley MS, Marciniak C, Eckel-Mahan K, McManus M, Crimi M, Waymire K, Shi Lin C, Masubuchi S, Friend N, Koike M, Chalkia D, MacGregor G, Sassone-Corsi P, Wallace

DC (2012) Heteroplasmy of mouse mtDNA is genetically unstable and results in altered behaviour and cognition. *Cell* **151**, 333-343.

Speijer D, Lukes J, Elias M (2015) Sex is a ubiquitous, ancient, and inherent attribute of eukaryotic life. *Proceedings of the National Academy of Sciences of the United States of America* **29**, 8827-8834.

Solignac M, Monnerot M, Mounolou JC (1983) Mitochondrial DNA heteroplasmy in *Drosophila mauritiana*. *Proceedings of the National Academy of Sciences of the United States of America* **80**, 6942-6946.

Stewart JB, Freyer C, Elson JL, Wredenberg A, Cansu Z, Trifunovic A, Larsson NG (2008) Strong purifying selection in transmission of mammalian mitochondrial DNA. *PloS Biology* **6**: e10.

Van Leeuwen T, Vanholme B, Van Pottelberge S, Van Nieuwenhuyse P, Nauen R, Tirry L, Denholm I (2008) Mitochondrial heteroplasmy and the evolution of insecticide resistance: Non-Mendelian inheritance in action. *Proceedings of the National Academy of Sciences of the United States of America* **105**, 5980-5985.

Welch ME, Darnell MZ, McCauley DE (2006) Variable Populations within variable populations: quantifying mitochondrial heteroplasmy in natural populations of the gynodioecious plant *Silene vulgaris. Genetics* **174**, 829–837.

Williams CC, Jan CH, Weissman JS (2014) Targeting and plasticity of mitochondrial proteins revealed by proximity-specific ribosome profiling. *Science* **346**, 748-751.

Wilson AJ, Xu J (2012) Mitochondrial inheritance: diverse patterns and mechanisms with an emphasis on fungi. *Mycology* **3**, 158-166.

Wolff JN, Nafisinia M, Sutovsky P, Ballard JWO (2013) Paternal transmission of mitochondrial DNA as an integral part of mitochondrial inheritance in metapopulations of *Drosophila simulans*. *Heredity* **110**, 57-62.

Wolff JN, Ladoukakis M, Enriquez JA, Dowling DK (2014) Mitonuclear interactions: Evolutionary consequences over multiple biological scales. *Philosophical Transactions of The Royal Society B Biological Sciences* **369**: 1646.

Woloszynska M (2010) Heterplasmy and stoichiometric complexity of plant mitochondrial genomes—though this be madness, yet there's method in't. *Journal of Experimental Botany* **61**, 657-671.

Woodson JD, Chory J (2008) Coordination of gene expression between organellar and nuclear genomes. *Nature Reviews: Genetics* **9**, 383-395.

2.7 | FIGURES

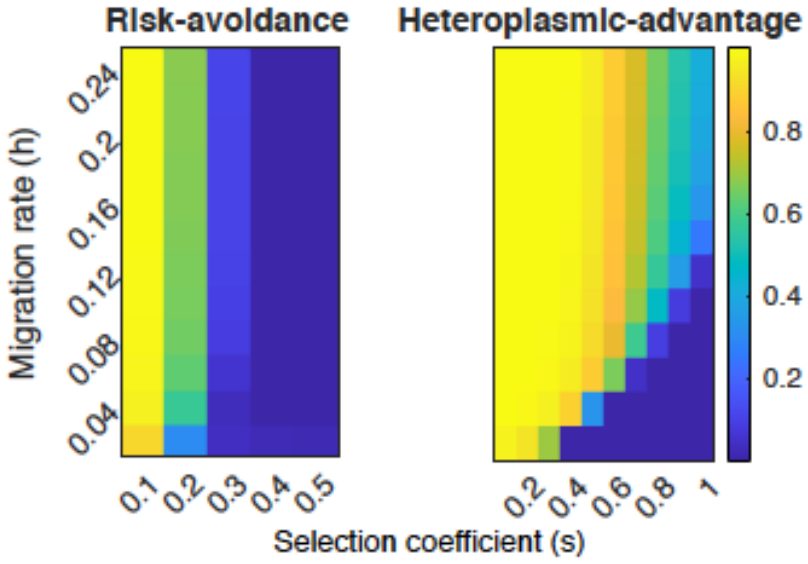

Figure 1. Mean frequency of the *u* allele throughout the entire cline at equilibrium or after 75000 generations; whichever came first. The *u* allele is injected into a metapopulation of hybridising cells at a frequency of 0.01 after migration/selection equilibrium has been reached along the cline. It is then able to evolve freely. For the parameter space explored, *u* invades more readily under the heteroplasmic advantage model than the risk-avoidance model. Yellow represents a high equilibrium frequency of *u* and blue represents little or no invasion, as denoted by the scale on the right hand side of the figure. In each case, the conditions where *u* reaches a high equilibrium frequency correspond to areas of low selection and higher migration, where gene flow is high. Parameter values: diploid mitochondrial number M = 100, number of demes C = 50.

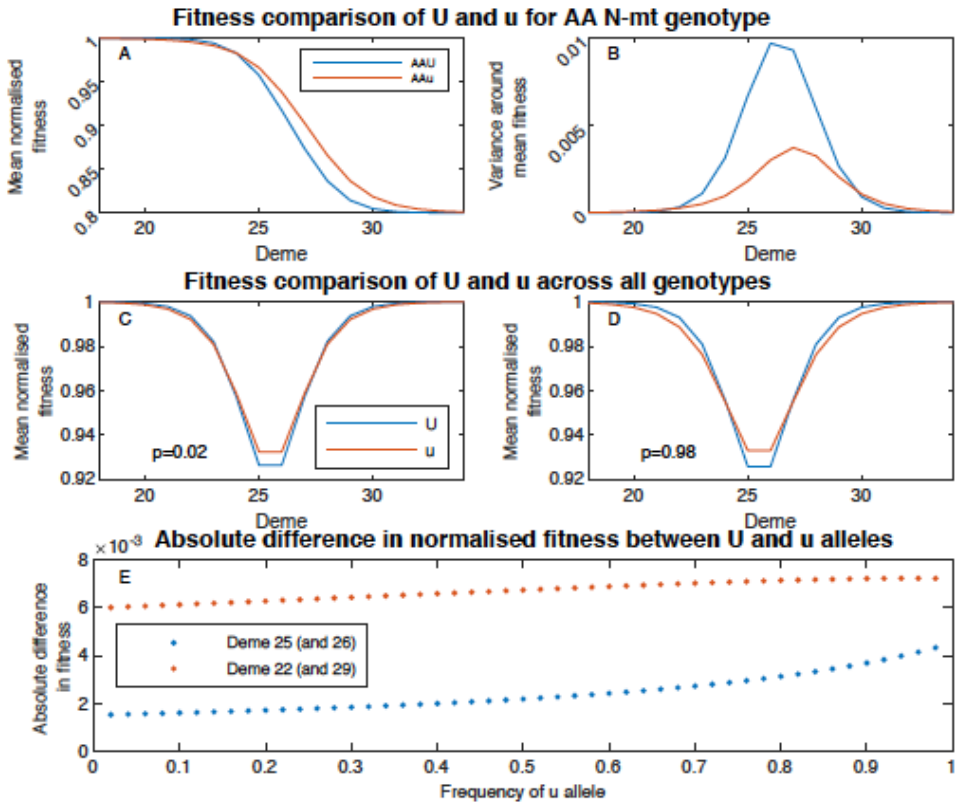

Figure 2. Comparison of the fitness of *U* and *u* individuals aggregated over specific N-mt genotypes and over the entire population. (A) In regions of the cline where the ***A*** allele is more common, *u* carries a cost to ***AA*** individuals and in regions of the cline where the ***A*** allele is less common, *u* provides an advantage. (B) *u* individuals have lower variance in fitness in the cline centre since they

have a much lower chance of producing either a mismatched 'low fitness' genotype or a fully matched 'high fitness' genotype. (C,D) Mean normalised fitness of the *U* and *u* alleles averaged across all genotypes under low frequency of *u* (C) and high frequency of *u* (D). *u* offers a net advantage in the middle two demes (25 and 26) and a disadvantage in the demes either side of the centre. This trend is exaggerated as the frequency of *u* increases (from panel C to D). While the difference in the cline centre increases roughly linearly with increasing frequency of *u* (red line, panel E), the difference outside the cline centre increases quadratically (blue line, panel E). Parameter values: $M = 100$, $C = 50$, migration rate $h = 0.1$, selection strength $s = 0.2$ (moderate gene flow). Fitness curves: 'risk-avoidance'.

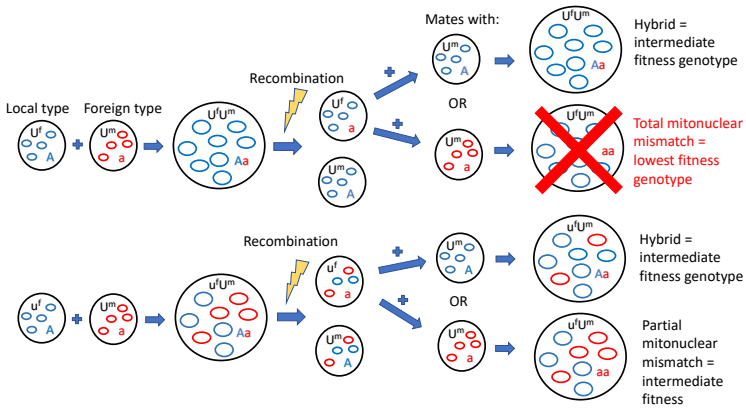

Figure 3. A schematic representation of how, through recombination, an ***A****U* gamete in its 'home range' risks eventually producing a totally mismatched ***aa****U* zygote when paired with a rare ***a*** allele. This is contrasted with the lower variance pathway of an ***A****u* gamete following the same path but producing descendants all of intermediate fitness. This scenario is played out in the '***A*** side' of the cline, where ***A*** and 1 haplotypes (represented by blue circles) are the most common and ***a*** and 0 haplotypes (red circles) are relatively rare.

CHAPTER III | Effects of mtDNA, nuclear genotype and their interaction on bioenergetic function in *Drosophila melanogaster.*

Tom Allison, Nicolas Pichaud, Craig R. White, Rebecca Koch, Damian K. Dowling

3.1 | ABSTRACT

The mitochondrial genomes of bilaterian metazoans are thought to harbour variation that affects organismal phenotype through their influence over core physiological traits such as oxidative phosphorylation. These effects may be shaped by a range of factors, including the sequence of the nuclear genome with which the mtDNA haplotype is 'paired', along with other physiological states such as sex and age. There are theoretical grounds to believe that mitochondrial haplotype can affect organismal phenotype in an additive fashion *as well as* in conjunction with the nuclear genotype. Surprisingly, few studies have explicitly attempted to assess the relative contributions of mitochondrial and nuclear genotype and their interaction to variation in core mitochondrial functions such as oxidative phosphorylation when sequence divergence between mtDNA haplotypes is representative of divergence between mtDNA haplotypes in human populations. We assess bioenergetic parameters in a 3 x 3 panel of *Drosophila melanogaster* that possess orthogonal combinations of three distinct nuclear genotypes and three distinct mitochondrial haplotypes. Importantly, the haplotypes diverge by at most ~0.3% in protein-coding regions. We find that bioenergetic parameters segregate along two main dimensions of variation which roughly capitulate to 'coupled' (ATP producing) and 'uncoupled' (non-ATP producing) respiration. Nuclear genotype and mitochondrial haplotype significantly influence coupled respiration, with roughly equivalent effect sizes that were each larger than the effect size of the (non-significant) mitonuclear interaction. Nuclear genotype and a mitonuclear interaction shape uncoupled respiration, with the magnitude of the nuclear genotype effect substantially outweighing the effect size of the interaction.

3.2 | INTRODUCTION

Eukaryotes depend on energy produced through oxidative phosphorylation (OxPhos) in mitochondria (Lane & Martin 2010; Lane 2015). The structure and function of the electron transport system (ETS) upon which OxPhos takes place depends on products encoded by two genomes – the nuclear and the mitochondrial – an enduring consequence of the endosymbiotic merger between two free-living prokaryotes that gave rise to eukaryotes (Margulis 1967; Martin & Muller 1998; Ryan & Hoogenraad 2007; Bar-Yaacov *et al*. 2012). In modern eukaryotes, the nuclear genome encodes most of the proteins comprising the ETS (Zhang & Broughton 2013; Walker & Moraes 2022) and has been shown to harbour intraspecific genetic variation related to OxPhos function (Jumbo-Lucioni *et al*. 2012). In bilaterians generally, the mitochondrial genome (mtDNA) is much smaller, often housing only 13 protein-coding genes related to the ETS, 22 transfer RNAs genes and two ribosomal RNAs genes (Wolstenholme 1992; Taanman 1999), and it too is thought to harbour non-neutral sequence variation that shapes phenotypic variation including variation in OxPhos function (Ballard & Whitlock 2004; Dowling *et al*. 2008; Galtier *et al*. 2009; Dobler *et al*. 2014; Wolff *et al*. 2016). Furthermore, given the tight coordination of products encoded by the two genomes, it is believed that epistasis between the two genomes may contribute toward variation in animal phenotypes (Blier *et al*. 2001; Rand *et al*. 2004; Ballard & Whitlock 2004; Dowling *et al*. 2008). The roles of nuclear genetic variation, mtDNA variation, and their interaction in shaping the trajectory of evolution in wild populations will depend on their relative contributions to variation in organismal phenotype; likely mediated by OxPhos function.

The empirical recognition that mitochondrial genomes carry functional (i.e. phenotype changing) variation came as a recent shift from the classic view that mitochondrial genomes should be purged of functional variants by a combination of strong purifying selection and uniparental inheritance (Birky 2001; Ruiz-Pesini *et al*. 2004; Meiklejohn *et al*. 2007; Ballard & Kreitman 1995; Wallace *et al*. 1999; Ballard & Whitlock 2004; Rand *et al*. 2004; Dowling *et al*. 2008; Dobler *et al*. 2014). There is now a wealth of experimental evidence linking mitochondrial sequence variation to a range of traits across bilaterian metazoans, including fertility and reproductive success (Aw *et al*. 2011, Camus *et al*. 2015, Camus & Dowling 2018), aging and longevity (James & Ballard 2003; Maklakov et al. 2006; Camus *et al*. 2012, Camus *et al*. 2015), development time (James & Ballard 2003), disease susceptibility (Fetterman *et al*. 2013, Betancourt *et al*. 2014), locomotor activity (Anderson *et al*. 2022), male sperm viability and courtship behaviour (Dowling et al. 2007a; Yee et al 2013, Koch & Dowling 2022), nuclear gene expression (Innocenti *et al*. 2011) and thermal tolerance (Balloux *et al*. 2009; Pichaud *et al*. 2013; Camus *et al*. 2017; Lajbner *et al.* 2018). It is presumed that much of the phenotypic variation in these traits is mediated by mtDNA effects on electron transport system structure and function (though recent research suggests that mitochondrial RNAs and mitochondria-derived micropeptides can also mediate mitonuclear genetic effects on phenotype (Miller *et al*. 2020; Pozzi & Dowling 2021; Breton 2021; Kienzle *et al*. 2023)). In line with this hypothesis, several studies have experimentally engineered strains of *Drosophila* and mice in which diverse mtDNA haplotypes are placed onto a common nuclear background, and subsequently showed that mitochondrial genetic variation is responsible for significant variation in core bioenergetic parameters (Katewa & Ballard 2007; Pichaud *et al*. 2012, Correa *et al*. 2012, Pichaud *et al*. 2013, Fetterman *et al.* 2013, Wolff *et al*. 2016, Camus *et al*. 2023). These effects may in turn

depend on several key physiological covariates, including age and sex (Wolff *et al*. 2016), thus highlighting the context dependency of mtDNA effects which must be accounted for in assessments of mtDNA functional variation on OxPhos.

It has been argued that one of the most important covariates moderating the effects of mitochondrial genetic variation on OxPhos and organismal function is the nuclear genome (Rand *et al*. 2001; Ballard & Whitlock 2004; Dowling *et al*. 2008). The fact that OxPhos function depends on tight coordination between products from both the mitochondrial *and* the nuclear genomes is thought to underly the signatures of mitonuclear genetic co-evolutionary adaptation that have been observed across animal taxa (Blier *et al*. 2001; Osada & Akashi 2012; Barreto & Burton 2013; Wang *et al*. 2021). Specifically, high mutation rates in mtDNA and the high ratio of non-synonymous to synonymous mutations (dN/dS) in nuclear genes related to OxPhos function suggest that the two genomes tend to evolve in lockstep, with the nuclear genome 'compensating' for functional mtDNA mutations. If mitochondrial mutations are impactful enough to force compensatory responses in the nuclear genome, we may expect that disrupting co-evolved pairs is likely to cause disrupted OxPhos function. Indeed, the results of several studies have supported this prediction. In both wild and experimental inter- and sub-species hybrid crosses, inviability and mortality is common (Ellison & Burton 2006; Burton *et al*. 2006, Niehuis et al 2008, Lee et al. 2008; Spirek et al 2014; Lamelza & Ailion 2017). At the intraspecific level, experimentally engineered and putatively novel combinations of mitochondrial and nuclear genotypes reveal that the mitonuclear interaction shapes the expression of a range of key fitness components including (but not limited to) development rate, reproductive success and lifespan (Clark & Lyckegaard 1988; Rand *et al.* 2001; Dowling et al. 2007b; Montooth *et al.*

2010; Camus *et al.* 2020; Rank *et al*. 2020; Immonen *et al*. 2016a,b; Rand *et al*. 2006, Meiklejohn *et al*. 2013; Dong *et al* 2019; Carnegie *et al.* 2021; Mossman *et al* 2016 a,b; Sujkowski *et al* 2019).

To understand how the interaction between mitochondrial and nuclear genetic variation affects OxPhos function, researchers have historically assayed mitonuclear hybrids created from greatly diverged lineages. Early research demonstrated complete failure of electron transport system function with interspecific crosses including human/chimpanzee and mice/rat mitonuclear hybrids (Barrientos *et al*. 1998; Dey *et al*. 2000). More recently, the intertidal copepod *Tigriopus californicus* has proved to be a fruitful model for studying the links between intraspecific mitonuclear interactions and function of individual electron transport system complexes (Ellison & Burton 2006). Recombinant crosses of distinct populations show reduced oxygen consumption at enzymatic complexes of the ETS that are constructed with protein products from both genomes (CI and CIV), but not at complexes entirely constructed with nuclear encoded proteins (CII) (Ellison & Burton 2006). Similar results have been found for hybrid crosses of fruit-flies, with crosses between distinct combinations of *Drosophila mauritiana, Drosophila simulans* and *Drosophila melanogaster* yielding varying forms of bioenergetic dysfunction (Sackton *et al*. 2003; Pichaud *et al*. 2019). Wild hybrid crosses of the mummichog fish *Fundulus heteorclitus* have also been shown to have compromised oxidative phosphorylation (Baris *et al*. 2017). The conclusions of these studies suggest that the interaction between mitochondrial and nuclear genomes might be the key moderator of mitochondrial bioenergetic function. In each case, however, sequence divergence between source populations is large (up to 20% in the case of *T. Californicus*) (Burton & Feldman 1981, Edmands 1999) and thus not representative of the kind of

mitochondrial and nuclear sequence divergence typical of wild, interbreeding populations of most species.

Partitioning variance attributable to each genome in systems that possess the kind of genetic divergence common in wild populations will give deeper insights into how often we might expect to observe functional mitonuclear epistasis and how impactful it will be in shaping evolution of populations. If the magnitude of the mitonuclear interaction depends in some way on the level of genetic divergence between interbreeding populations, then the partitioning of variance in OxPhos function across nuclear and mitochondrial genomes and their interaction in mitonuclear hybrid models is likely not representative of the way variance is partitioned in wild, interbreeding populations. In other words, additive mtDNA effects that may be important in shaping OxPhos phenotype in wild populations could be obscured under such enormous nuclear divergence where mitonuclear epistasis dominates. Indeed, the overwhelming amount of evidence appearing in support of mitonuclear epistasis should not be taken as an indication that additive mtDNA variation doesn't contribute to phenotypic variation. We argue that when assessing the scope of the mitonuclear interaction in shaping evolutionary patterns in wild populations, it is important to quantify the relative contributions of nuclear and mitochondrial genetic variation as well as their interaction, and that this should be assessed under levels of genetic divergence common to wild, interbreeding populations. Furthermore, given the context dependency of mtDNA effects and mitonuclear interactions on environmental and physiological variables (Zhu *et al*. 2014; Mossman *et al*. 2016a; Mossman *et al*. 2016b; Wolff *et al*. 2016; Pichaud *et al.* 2019), a thorough attempt to partition variance in OxPhos function according to mtDNA and nuclear variation would account for important covariates such as age and sex.

Here, we sought to address two questions. Firstly, are mitochondrial genetic effects on OxPhos consistent despite pairing with nuclear genomes that diverge from one another with a magnitude representative of commonly interbreeding populations? Secondly, if nuclear genome and mtDNA both affect OxPhos, and so does their interaction, how do the effects of each compare in magnitude? To address these questions, we tested whether strains of fruit-fly (*Drosophila melanogaster*) genetically engineered to possess unique and orthogonal combinations of mitochondrial and nuclear genotypes differed in various high-resolution measurements of ETS function. We aimed to assay a range of states of ETS function that would accurately reflect the full range of possible states *in vivo*. We included individual measures of respiratory complexes I-III in the coupled (ATP producing) state, as well as maximum OxPhos capacity in the coupled state. We also took measurements of uncoupled (non-ATP producing) respiration, which allowed us – in combination with measures of coupled respiration – to calculate the respiratory control ratio (RCR), a gauge of the bioenergetic efficiency of mitochondria calculated as the ratio of oxygen that is consumed when ATP synthase is functioning compared to when it is inhibited. This metric gives an indication of the relative amount of oxygen required to sustain membrane potential as hydrogen ions 'slip' from the intermembrane space into the mitochondrial matrix through the inner mitochondrial membrane (rather than through ATP synthase) (Brand & Nicholls 2011). Lower RCR values indicate that mitochondria are producing ATP with reduced efficiency, and RCR has therefore been put forward as one of the most important metrics of mitochondrial dysfunction (Brand & Nicholls 2011). Including RCR in our assay should yield the highest chance of exposing mitochondrial and nuclear genetic incompatibilities, which are predicted to manifest as reduced RCR.

Flies were sourced from three allopatric populations of *D. melanogaster* that are estimated to possess mtDNA sequence divergence within protein-coding regions of approximately ~0.1-0.3% (Morrow *et al*. 2015). The full-factorial panel of replicated mitonuclear strains was created through repeated paternal backcrossing, which allowed for matching of three distinct and naturally occurring mitochondrial haplotypes alongside three divergent and near-isogenic target nuclear backgrounds. This framework afforded us the ability to partition mitochondrial and nuclear genetic main effects, as well as isolate subtle mitonuclear interactive effects from potentially dominant nuclear-nuclear interactive effects. We assayed male and female flies separately, and – for each sex and strain – assayed flies at two different ages (day 5 and day 15).

3.3 | MATERIALS & METHODS

3.3.1 | Mitonuclear strains

The flies used in this study were originally sourced from three allopatric populations of *D. melanogaster*; MAD (Madang, Papua New Guinea), PUE (Puerto Montt, Chile) and ZIM (Zimbabwe) (Clancy 2008; Camus *et al*. 2012). Since then, they have been maintained as near-isogenic female lines in a laboratory environment for approximately 250 generations. Isogenicity was initially ensured by propagating each line through one full sibling mating pair per generation, for at least 10 generations. Lines were maintained as near-isogenic through full sibling crosses within each line. Using these lines, a fully factorial panel of all possible mitochondrial/nuclear genotypes was created through selective introgression of the three

mitochondrial haplotypes onto each of the three nuclear backgrounds. In the first generation, in January 2018, 5 males from each nuclear background were paired with 5 virgin females from distinct lines possessing the intended mitotype and a *w1118* nuclear background which had been established earlier (Clancy 2008; Camus *et al*. 2012). Each subsequent generation, males possessing the intended nuclear background were crossed with virgin females possessing the intended mitochondrial haplotype, sourced from the previous generation of backcrossing. At the time flies were taken for bioenergetic assay, the PUE strains had undergone 60 generations of introgressive backcrossing, and the MAD and ZIM strains had undergone 63 generations. The result is nine unique and controlled combinations (hereafter 'strains') of mitochondrial and nuclear genotype, with three of the nine strains exhibiting population-specific combinations of mito-nuclear genotype that were putatively 'matched' and the remainder having putatively 'mismatched' combinations. Each of these strains were initially created and subsequently maintained in duplicate, for a total of 18 strains (nine mito-nuclear genotypes, with two replicates per genotype).

3.3.2 | Fruit-fly maintenance and preparation

Due to limited throughput of the bioenergetics assay, we did not sample both duplicate sets of each mitonuclear genotype, and instead admixed flies from each duplicate set two generations before sampling (hereafter the 'grandparental generation'). Their offspring were then bred among themselves (full-sibling crosses) to produce the focal generation for assay. This process was repeated in four separate sampling blocks, each temporally separated by non-consecutive generations of fly culture. This approach precluded assessment of within-genotype variation across the strains, since duplicate strains of each genotype were combined for the assay. However, admixing the two duplicates provides, at

least in theory, a more accurate representation of the mean phenotype for each genotype compared to sampling either duplicate in isolation. This is a consequence of the law of large numbers, which holds that the average of any two samples is, statistically, more likely to be closer to the true population mean than either of the samples alone.

For each sampling block, 32 pairs of grand-parental flies were collected per mitonuclear genotype across the two duplicates. They were allocated across two vials (16 pairs per vial) containing 6mL food media (mixed from potato, dextrose, yeast and agar, sprinkled with ad lib live yeast granules and treated to prevent mould) and were allowed to mate and oviposit for 24 hours. Excess eggs were trimmed to a target density of 100. At the grand-parental generation, adult pairs from each strain were transferred into fresh vials after each 24h period to enable females to lay eggs on the food substrate of each vial for a total of five consecutive 24h periods (hereafter termed a 'lay'). This process yielded five technical replicates of each strain that were identical in all respects other than the age of their parents. Thirteen days later, at day three of age post-eclosion, 32 progeny from the grandparental flies (16 pairs) were collected from each strain, allocated across three vials, and allowed to mate and oviposit for 24h.

At eclosion (10 days after each lay), sixty virgin flies per sex were collected per strain, per lay and allocated across three vials (20 per vial) to minimise density effects. The result was a set of five staggered focal groups, each of the same sex, age and mitonuclear strain, separated only by the age of their grandparents (with a maximum of 5 days difference between the first and last lay). Focal flies were maintained until assayed at ages 5- and 15-days, with each focal group transferred to new vials containing fresh food every three days, up to the point

of assay. At all times, flies were housed at constant temperature (25.0 ± 1 ^{o}C) and exposed to a diurnal light cycle (12:12h light/dark).

3.3.3 | High resolution respirometry measurements

Flies of each sex and strain were sampled for assay at ages 5- and 15-days. Only thorax muscle tissue was sampled, as it the tissue containing the highest density of mitochondria in fruit flies. For measurement of respiration, we followed the methods of Simard et al (2018). Briefly, thorax muscle (3 thoraces per sample for male flies, and 2 thoraces per sample for female flies) was permeabilized at 4 °C using BIOPS relaxing solution (2.77mM CaK_2EGTA, 7.23mM K_2EGTA, 5.77 mM Na_2ATP, 6.56mM $MgCl_2$, 20mM taurine, 15mM Na_2phosphocreatine, 20mM imidazole, 0.5mM dithiothreitol, 50mM K-MES, pH 7.2) complemented with 62.5µg/mL saponin, and subsequently blot-dried, weighed and transferred into the respiration chamber of an Oxygraph-2k respirometer (Oroboros Instruments, Innsbruck, Austria), calibrated with air-saturated respiration medium (115 mM KCl, 10 mM KH_2PO_4, 2 mM $MgCl_2$, 3 mM HEPES, 1 mM EGTA, 0.2% BSA, pH 7.2) at 25 °C. Prior to the transfer of fibres, pyruvate and malate (10 mM each) were added to the respiration medium in order to stimulate the NADH-pathway without oxidative phosphorylation (N-LEAK). Thorax tissue fibres were then transferred into respiration chambers and N-LEAK respiration was monitored. Next, we added ADP (5 mM) to measure oxidative phosphorylation sustained only by complex I substrates (N-OXPHOS). Next, we assessed the integrity of the outer mitochondrial membrane through addition of cytochrome c (15 µM), with samples showing a rise of ≥15% in oxygen consumption discarded, as per Simard *et al*. (2018). We then added sequential injections of the following substrates: succinate (10 mM) to stimulate complex II (NS-OXPHOS), proline (10 mM) to fuel

proline dehydrogenase (NSPro-OXPHOS), and *sn* glycerol-3-phosphate (20 mM) to stimulate glycerol-3-phosphate dehydrogenase and induce maximum oxidative phosphorylation (max-OXPHOS). After measurement of max-OXPHOS, oligomycin (0.01 µM) was added to inhibit the function of ATP synthase. This prevents oxidative phosphorylation, so the resultant oxygen respiration – termed State IV – is attributable entirely to maintaining the electrochemical gradient as H+ ions slip back across the mitochondrial inner membrane.

Once the State IV signal had levelled, rotenone (1 µM), malonate (10 mM) and antimycin A (2.5 µM) were added to inhibit complex I, complex II and complex III respectively, allowing for measurement of residual oxygen consumption. Finally, N,N,N',N'-tetramethyl-p-phenylenediamine (TMPD, 0.5 µM) and ascorbate (2 mM) were added to measure complex IV activity (COX), which was corrected for chemical background oxidation after complete inhibition of COX by sodium azide (20 mM). Our calculation for the respiratory control ratio was simply max-OXPHOS divided by State IV respiration (Brand & Nicholls 2011)

All measurements are expressed as means of respiration rates in picomol of oxygen consumed per second and per mg of thorax tissue (pmol s^{-1} mg^{-1}). Data was collected for a total of 286 samples, evenly distributed across the nine strains, both sexes and two ages.

3.3.4 | Statistical Analysis

All analyses were conducted using R software (4.2.0).

Our goal was to capture any differences in the major axes of bioenergetic variation. Given the high correlations between oxygen consumption across various states of ETS stimulation,

we reduced the dimensionality of the response variables through a principal component analysis. Of a total 2002 measurements across 286 samples, 19 values were missing. We imputed these values using multiple imputation, which involves using variation in the known values to estimate missing values and repeating this process multiple times to generate valid statistical inference (Kang 2013; Sterne *et al*. 2009; Jakobsen *et al*. 2017; Hayati Rezvan *et al*. 2015).

PC1, PC2 and RCR values were fit to general linear mixed models, with nuclear genotype, mitochondrial haplotype, sex, age and their interactions (limited to three-way interactions) included as fixed effects and respirometer chamber (four distinct chambers were used in sampling) and block treated as random effects. Residual maximum likelihood (REML) was used to estimate the variance of the model parameters. F-tests with Kenward-Rogers approximation of degrees of freedom, and a type III model with sum-to-zero contrasts were used to assess the significance of fixed terms. Stepwise removal of non-significant, fixed interaction terms was used to reduce the model to a final model containing only main effects and significant interactions. Terms, beginning with least-significant three-way interactions and moving to two-way interactions, were removed if and only if log likelihood ratio tests showed a significant difference in the residual deviance in the model after exclusion of the candidate term. The mitonuclear interaction was retained in models regardless of its significance since modelling estimates around its effect size was a primary goal of the present study.

To estimate effect sizes for each term in our models, we extracted partial omega squared (ω^2) statistics, along with 95% confidence intervals. The ω^2 statistic estimates the

proportion of variance in PC1 and PC2 that is accounted for by each term. We chose ω^2 as it is considered more robust to bias when compared to commonly used measures of effect size such as eta-squared (Keselman 1975; Troncoso-Skidmore & Thompson 2013).

3.4 | RESULTS

3.4.1 | Principal Component Analysis results

Principal component 1 (PC1) accounted for 52.7% of the variance in the data set, with respiratory parameters representing coupled (OXPHOS) respiration (N-OXPHOS, NPro-OXPHOS, NProS-OXPHOS, max-OXPHOS) loading heavily onto this axis (Figure 1). Higher PC1 scores are representative of high oxygen consumption during coupled respiration. The next principal component (PC2) accounted for 21.5% of the variance and was best described with parameters that represent uncoupled respiration through either ADP insufficiency or ATP synthase inhibition (N-LEAK, State IV and COX) (Figure 1). Higher PC2 scores are representative of lower oxygen consumption during uncoupled respiration.

3.4.2 | Sources of variance affecting principal components

Our measure of coupled respiration, PC1, was significantly affected by variation in all four main effects, including nuclear genotype ($F_{2,272} = 3.15$, $p = 0.044$), mitochondrial genotype ($F_{2,272} = 4.52$, $p = 0.011$), sex ($F_{1,270} = 44.38$, $p < 0.001$) and age ($F_{1,270} = 40.15$, $p < 0.001$). The interaction between mitochondrial and nuclear genotype did not contribute significantly toward variation in PC1. Age (ω^2=0.13, 95% CI [0.06-0.20]) and sex (ω^2=0.14, 95% CI [0.07-

0.22]) exhibited the largest effect sizes, with nuclear genotype (ω^2=0.02, 95% CI [0.00-0.05]) and mitochondrial haplotype (ω^2=0.03, 95% CI [0.00-0.07]) following. The PUE nuclear genotype was associated with higher rates of OxPhos than MAD and ZIM nuclear genotypes (Fig. 2a). The MAD mtDNA haplotype consistently showed lower rates of OxPhos (Figure 2b). Female flies showed higher OxPhos rates than males (Figure 2c), as did older flies compared to younger flies (Figure 2d).

PC2, our measure of oxygen consumption in the absence of ATP synthase activity, was significantly affected by nuclear genotype ($F_{2,264}$ = 69.10, p < 0.001), with a large associated effect size (ω^2=0.34, 95% CI [0.25-0.42]). PC2 scores were also subject to a significant interaction between mitochondrial and nuclear genotypes ($F_{4,263}$ = 2.58, p = 0.038), which was associated with a smaller effect size (ω^2=0.02, 95% CI [0.00-0.06]). This interaction was caused by a lack of mtDNA haplotype effect in the Madang and Puerto Montt nuclear backgrounds, but an mtDNA effect in the Zimbabwe background, whereby the Zimbabwe haplotype exhibited lower PC2 values (and therefore higher uncoupled respiration) than the other haplotypes when expressed alongside the putatively matched Zimbabwe nuclear background (Figure 3a). Sex and age were again significant determinants of PC2 scores ($F_{2,264}$ = 69.10, p < 0.001, $F_{1,265}$ = 89.77; p < 0.001, $F_{1,262}$ = 38.96, p < 0.001 respectively), and were responsible for large effects (ω^2=0.25, 95% CI [0.27-0.34], ω^2=0.13, 95% CI [0.06-0.21] respectively). In addition to their influence as main effects, PC2 was affected by an interaction between nuclear genotype, sex and age (Nuclear x Sex x Age; $F_{2,264}$ = 3.73, p = 0.025), though this was associated with a small effect size (ω^2=0.02, 95% CI [0.00-0.06]). The interaction appears to be driven by 15-day old males of the Puerto Montt nuclear

genotype, which show low PC2 scores for their age. This suggests that these males have high levels of uncoupled respiration that continues into later life (Figure 3b,c).

RCR, our measure of respiratory efficiency, was significantly affected by nuclear genotype ($F_{2,267}$ = 57.82, p < 0.001), sex ($F_{2,267}$ = 209.98, p < 0.001) and age ($F_{2,265}$ = 136.43, p < 0.001). Effect sizes for nuclear genotype (ω^2=0.30, 95% CI [0.21-0.38]), sex (ω^2=0.44, 95% CI [0.36-0.51]) and age (ω^2=0.34, 95% CI [0.25-0.42]) were all large. RCR scores were also subject to significant interactions between nuclear genotype and age ($F_{4,266}$ = 5.12, p = 0.007) and sex and age ($F_{4,265}$ = 26.22, p <0.001). These interactions were responsible for small and moderate effect sizes respectively (ω^2=0.03, 95% CI [0.00-0.08] and ω^2=0.09, 95% CI [0.03-0.16]).

3.5 | DISCUSSION

We demonstrate that major dimensions of variation in OxPhos function are significantly influenced by nuclear genotype, mitochondrial haplotype, and the interactive effect between the two. Furthermore, we show that the amount of variance in OxPhos function attributable to main effects of the nuclear or mitochondrial genomes variation is greater in magnitude than the variance attributable to the mitonuclear interaction. Importantly, these findings are obtained from a fully factorial panel of *D. melanogaster* where divergence within the protein-coding regions of mtDNA haplotypes is comparable to levels of divergence commonly seen between wild, interbreeding populations.

Nuclear genotype appears to have the most widespread effects on OxPhos function. Nuclear genotype is partly responsible for variation in our metrics of both coupled and uncoupled respiration, as well as respiratory control ratio, acting in both an additive fashion and in concert with age and sex to determine OxPhos phenotype. The strong effects of nuclear genotype likely reflect the absolute abundance of nuclear encoded mitochondrial genes (Calvo *et al*. 2016), which, in aggregate, allow for substantial standing variation across populations even in the face of strong purifying selection on mitochondrial function (Rowe & Houle 1996). Nuclear control over uncoupled respiration may be mediated by variation in uncoupling proteins. These proteins are embedded in the mitochondrial inner membrane and control the rate of proton movement from the inter-membrane space back into the mitochondrial matrix (Ledesma *et al*. 2002; Brand & Esteves 2005). Importantly, they are known to be nuclear encoded – at least in animals (Ledesma *et al* 2002). Variation in uncoupled respiration could be linked to variation in either uncoupling protein structure or expression/regulation (Ledesma *et al*. 2002), and this remains an avenue for future work. Nuclear genotypic effects over RCR are likely a reflection of the nuclear involvement in both coupled and uncoupled respiration, since RCR describes the ratio of maximum coupled respiration to uncoupled LEAK respiration. However, nuclear genetic control over RCR is not a trivial consequence of nuclear control over both coupled and uncoupled respiration rates. This is because nuclear genotype could influence each of these parameters in such a way that their ratio is preserved. The fact that the nuclear genotype influences RCR, as demonstrated in the present study, suggests that nuclear genotype controls coupled and uncoupled respiration rates somewhat independently – in turn suggesting that selection may act on each trait separately, or on the ratio itself.

The present study builds on earlier work linking mtDNA variation to OxPhos function (Pichaud *et al*. 2012, Correa *et al*. 2012, Wolff *et al*. 2016) by showing that mitochondrial haplotype shows additive effects when paired with divergent nuclear backgrounds. Regarding coupled respiration, we note that the estimated effect size of mitochondrial haplotype is comparable to that of the nuclear genotype. The equivalent effect size of mitochondrial and nuclear genotype is an unusual finding in comparison to prior research focussing on primary fitness components such as development time, egg-to-adult viability and longevity, which often show much larger summary statistics associated with nuclear genotype than mitochondrial haplotype (Clancy 2008; Mossman *et al*. 2016a; Drummond *et al*. 2019; Carnegie *et al*. 2021). Our work highlights the intuitive result that the relative contribution of each genome depends on the trait in question, with some traits directly related to mitochondrial function, such as coupled respiration, showing mitochondrial and nuclear genotypic effects that are comparable in magnitude.

In our study system, the main effects of both mtDNA and nuclear genome variation were more influential on bioenergetic phenotypes than the mitonuclear interaction. One possible explanation is that variation in mtDNA is largely adaptive, and does not require strong nuclear compensatory evolution. Indeed, a recent analysis suggests that across bilaterian metazoans, 26% of non-synonymous substitutions in mtDNA are adaptive, with the remainder being neutral or weakly deleterious (James *et al*. 2016). A combination of site-specific positive selection and strong, widespread purifying selection is thought to underly this pattern (Stewart *et al*. 2008; Morales *et al*. 2015). Indeed, multilevel selection, selection across ontogeny and repeated bottlenecking of mtDNA may allow for extremely efficient purifying selection on deleterious substitutions (Cree *et al*. 2008; Fan *et al*. 2013; Hill *et al*.

2019; Stewart & Larsson 2014; Camus & Dhawanjewar 2023). Efficient purifying selection on mtDNA leaves fewer deleterious mtDNA mutations at mutation-selection equilibrium, placing weaker selective pressures on the nuclear genome to compensate. By showing that additive mtDNA variation contributes more substantially to coupled respiration than the mitonuclear interaction, our work supports a model whereby mtDNA additive variation can accumulate since the adaptive or weakly deleterious mutations that remain after purifying mtDNA selection do not place strong selection pressures on compensatory nuclear changes.

Although a large portion of the important variation in the mitochondrial genome may act in an additive fashion, some variation evidently depends on the nuclear genome for its expression. We show that sequence divergence up to 0.3% within the protein coding regions of mtDNA is sufficient to induce mitonuclear interactions for uncoupled respiration. Given that this level of divergence is likely common across interbreeding populations in the wild (Morrow *et al*. 2015), our findings suggest that selection may frequently act to favour or penalise specific combinations of mitochondrial and nuclear genomes. Curiously, despite a mitonuclear interaction affecting uncoupled respiratory rate, mitochondrial genes do not encode uncoupling proteins that are thought to regulate membrane permeability (and consequently uncoupled respiration rates). One possible way for mitochondria to work in concert with the nuclear genome to affect uncoupled respiration is through retrograde signalling. If nuclear genetic variation exists in uncoupling proteins, and differences in regulatory signalling thresholds exist across mitochondrial haplotypes, we may expect to observe a pattern of epistasis controlling uncoupled respiration rates. Indeed, although UCPs are known to be nuclear encoded, their expression is thought to be regulated within the mitochondrion (Ledesma *et al*. 2002). Our work suggests that strong selection on rates

of uncoupled respiration (as opposed to coupled respiration) may lead to tighter mitonuclear co-evolution. This may be a substantial contributing factor toward the variation in mitochondrial haplotypes observed across human populations, as climate is thought to place strong selective pressures on uncoupled respiration rates due to its thermogenic role in endotherms (Mishmar *et al*. 2003; Ruiz-Pesini 2004; Balloux *et al*. 2009).

Partitioning the effects of mtDNA, nuclear genotype and their interactive effect on OxPhos in the present study sheds light on the distribution of mitochondrial haplotypes in wild populations. It has been recognised that, contrary to a strong mitonuclear compatibility model, there is often genealogical discordance between mitochondrial and nuclear genotypes along species distributions (Riesenberg & Soltis 1991; Currat *et al*. 2008; Funk & Omland 2003; Toews & Brelsford 2012). One explanation for this trend is that mitochondrial additive genetic variation is substantial enough that populations of varying nuclear genealogies can improve fitness through introgression of specific haplotypes despite potential disruptions to co-evolved mitonuclear alleles (Sloan *et al*. 2017). The present study furnishes evidence for the plausibility of such cases of introgressive evolution, whilst also showcasing the mitonuclear interactive effects that may underpin alternative cases of strongly concordant mitochondrial and nuclear genealogies. Furthermore, our work suggests that whether a population experiences stronger selective forces on either coupled respiration or uncoupled respiration may influence the strength of mitonuclear coevolution within populations and thus whether they stand to benefit from introgression of 'fit' mitochondrial haplotypes. If selection is related to coupled respiration, we may expect additive effects of nuclear and mitochondrial genomes to predominate, and thus expose populations to mtDNA introgressive events. On the contrary, selective forces acting on

uncoupled respiration might lead selection to act on combinations of nuclear and mitochondrial genomes, thus leading to tight mitonuclear co-evolutionary processes. Assessing differences in coupled and uncoupled respiration across cases of mitonuclear genealogical concordance and discordance remains a fruitful avenue for future work.

In summary, we have demonstrated that the nuclear genome, the mitochondrial genome, *and* their interaction each influence different bioenergetic parameters. The nuclear genome tends to have the most widespread impact across various parameters and is associated with the largest effect sizes, compared to mitochondrial haplotype and the mitonuclear interaction. Mitochondrial haplotype appears to influence coupled respiration in an additive fashion, with an effect size equivalent to the nuclear genome. Thus, nuclear genotype, mitochondrial genotype and then their interaction appear to affect coupled respiration in that order of influence. Such a finding suggests that, while constituting a minority of substitutions, adaptive variation in mtDNA may be largely impactful, and may not place strong co-evolutionary pressures on nuclear genomes. However, the mitonuclear interaction is shown to influence uncoupled respiration, while mtDNA does not. The fact that different factors shape different respiratory parameters suggests that the partitioning of variance: the respective importance of nuclear DNA, mitochondrial haplotype and their interaction, may depend on the type of selection predominating within a population.

3.6 | REFERENCES

Anderson L, Camus MF, Monteith KM, Salminen TS, Vale PF (2022) Variation in mitochondrial DNA affects locomotor activity and sleep in Drosophila melanogaster. *Heredity* **129**, 225-232.

Aw WC, Correa CC, Clancy DJ, Ballard JW (2011) Mitochondrial DNA variants in Drosophila melanogaster are expressed at the level of the organismal phenotype. Mitochondrion **11**, 756-63.

Ballard JW, Kreitman M (1995) Is mitochondrial DNA a strictly neutral marker? Trends Ecol Evol. **10**, 485-488.

Ballard JW, Whitlock MC (2004) The incomplete natural history of mitochondria. Mol Ecol. **13**, 729-744.

Balloux F, Handley LJL, Jombart T, Liu H, Manica A (2009) Climate shaped the worldwide distribution of human mitochondrial DNA sequence variation. *Proc R Soc Biol Sci Ser B*. **276**, 3447-3455.

Baris TZ, Wagner DN, Dayan DI, Du X, Blier PU, Pichaud N, Oleksiak MF, Crawford DL (2017) Evolved genetic and phenotypic differences due to mitochondrial-nuclear interactions. *PLoS Genet*. **13**, e1006517.

Barreto FS, Burton RS (2013) Evidence for compensatory evolution of ribosomal proteins in response to rapid divergence of mitochondrial rRNA. *Molecular Biology and Evolution* **30**, 310-314.

Barrientos A, Kenyon L, Moraes CT (1998) Human xenomitochondrial cybrids. Cellular models of mitochondrial complex I deficiency. *J Biol Chem*. **273**, 14210-7.

Bar-Yaacov D, Blumberg A, Mishmar D (2012) Mitochondrial-nuclear co-evolution and its effects on OXPHOS activity and regulation. Biochim Biophys Acta **1819**, 1107-1111.

Betancourt AM, King AL, Fetterman JL, Millender-Swain T, Finley RD, Oliva CR, Crowe DR, Ballinger SW, Bailey SM (2014) Mitochondrial-nuclear genome interactions in non-alcoholic fatty liver disease in mice. *Biochem J*. **461**, 223-32.

Birky CW Jr (2001) The inheritance of genes in mitochondria and chloroplasts: laws, mechanisms, and models. *Annu Rev Genet*. **35**, 125-48.

Blier PU, Dufresne F, Burton RS (2001) Natural selection and the evolution of mtDNA-encoded peptides: evidence for intergenomic co-adaptation. *Trends Genet* **17**, 400–406.

Brand MD, Esteves TC (2005) Physiological functions of the mitochondrial uncoupling proteins UCP2 and UCP3. *Cell Metab.* **2**, 85-93.

Brand MD, Nicholls DG (2011) Assessing mitochondrial dysfunction in cells. *Biochem J.* **435**, 297-312.

Breton S (2021) Mitochondrial Russian doll genes may explain some discrepancies in links between mtDNA mutations and mitochondrial diseases. *BioEssays* **43**, 2100104.

Burton RS, Feldman MW (1981) Population genetics of *Tigriopus californicus*. II. Differentiation among neighboring populations. *Evolution* **35**, 1192, 1205

Burton RS, Ellison CK, Harrison JS (2006) The sorry state of F2 hybrids: consequences of rapid mitochondrial DNA evolution in allopatric populations. *Am Nat.* **168**, S14-24.

Calvo SE, Clauser KR, Mootha VK (2016) MitoCarta 2.0: an updated inventory of mammalian mitochondrial proteins. *Nucleic Acids Res*. **44**, 1251-7.

Camus MF, Clancy DJ, Dowling DK (2012) Mitochondria, maternal inheritance, and male aging. *Current Biology* **22**, 1717-1721.

Camus MF, Wolf JB, Morrow EH, Dowling DK (2015) Single Nucleotides in the mtDNA Sequence Modify Mitochondrial Molecular Function and Are Associated with Sex-Specific Effects on Fertility and Aging. Curr Biol. **25**, 2717-2722.

Camus MF, Wolff JN, Sgrò CM, Dowling DK (2017) Experimental Support That Natural Selection Has Shaped the Latitudinal Distribution of Mitochondrial Haplotypes in Australian Drosophila melanogaster. *Mol Biol Evol.* **34**, 2600-2612.

Camus MF, Dowling DK (2018) Mitochondrial genetic effects on reproductive success: signatures of positive intrasexual, but negative intersexual pleiotropy. *Proc Biol Sci*. **285**, 20180187.

Camus MF, O'Leary M, Reuter M, Lane N (2020) Impact of mitonuclear interactions on life-history responses to diet. *Philos Trans R Soc Lond B Biol Sci*. **375**, 20190416.

Camus MF, Dhawanjewar AS (2023) Multilevel selection on mitochondrial genomes. *Curr Opin Genet Dev*. **80**, 102050.

Camus MF, Rodriguez E, Kotiadis V, Carter H, Lane N (2023) Redox stress shortens lifespan through suppression of respiratory complex I in flies with mitonuclear incompatibilities. *Exp Gerontol*. **175**, 112158.

Carnegie L, Reuter M, Fowler K, Lane N, Camus MF (2021) Mother's curse is pervasive across a large mitonuclear *Drosophila* panel. *Evol Lett.* **5**, 230-239.

Clancy DJ (2008) Variation in mitochondrial genotype has substantial lifespan effects which may be modulated by nuclear background. *Aging Cell* **7**, 795-804.

Clark AG, Lyckegaard EM (1988) Natural selection with nuclear and cytoplasmic transmission. III. Joint analysis of segregation and mtDNA in Drosophila melanogaster. *Genetics* **118**, 471-81.

Correa CC, Aw WC, Melvin RG, Pichaud N, Ballard JWO (2012) Mitochondrial DNA variants influence mitochondrial bioenergetics in *Drosophila melanogaster*. *Mitochondrion* **12**, 459-464

Cree LM, Samuels DC, de Sousa Lopes SC, Rajasimha HK, Wonnapinij P, Mann JR, Dahl HH, Chinnery PF (2008) A reduction of mitochondrial DNA molecules during embryogenesis explains the rapid segregation of genotypes. *Nat Genet.* **40**, 249-54.

Currat M, Ruedi M, Petit RJ, Excoffier L (2008) The hidden side of invasions: massive introgression by local genes. *Evolution* **62**, 1908-20.

Dey R, Barrientos A, Moraes CT (2000) Functional constraints of nuclear-mitochondrial DNA interactions in xenomitochondrial rodent cell lines. *J Biol Chem*. **275**, 31520-7.

Dobler R, Rogell B, Budar F, Dowling DK (2014) A meta-analysis of the strength and nature of cytoplasmic genetic effects. J Evol Biol. **27**, 2021-2034.

Dong W, Dobler R, Dowling DK, Moussian B (2019) The cuticle inward barrier in *Drosophila* melanogaster is shaped by mitochondrial and nuclear genotypes and a sex-specific effect of diet. *PeerJ* **7**,e7802.

Dowling DK, Nowostawski AL, Arnqvist G (2007a) Effects of cytoplasmic genes on sperm viability and sperm morphology in a seed beetle: implications for sperm competition theory? *J Evol Biol* **20**, 358-68.

Dowling DK, Friberg U, Hailer F, Arnqvist G (2007b). Intergenomic epistasis for fitness: within-population interactions between cytoplasmic and nuclear genes in Drosophila melanogaster. *Genetics* **175**, 235-44.

Dowling DK, Friberg U, Lindell J (2008) Evolutionary implications of non-neutral mitochondrial genetic variation. Trends Ecol Evol. **23**, 546-554

Drummond E, Short E, Clancy D (2019) Mitonuclear gene X environment effects on lifespan and health: how common, how big? *Mitochondrion* **49**, 12–18.

Edmands S (1999) Heterosis and outbreeding depression in interpopulation crosses spanning a wide range of divergence. *Evolution* **53**, 1757-1768.

Ellison CK, Burton RS (2006) Disruption of mitochondrial function in interpopulation hybrids of Tigriopus californicus. *Evolution* **60**, 1382-91.

Fan W, Waymire KG, Narula N, Li P, Rocher C, Coskun PE, Vannan MA, Narula J, Macgregor GR, Wallace DC (2008) A mouse model of mitochondrial disease reveals germline selection against severe mtDNA mutations. *Science* **319**, 958-62.

Fetterman JL, Zelickson BR, Johnson LW, Moellering DR, Westbrook DG, Pompilius M, Sammy MJ, Johnson M, Dunham-Snary KJ, Cao X, Bradley WE, Zhang J, Wei CC, Chacko B, Schurr TG, Kesterson RA, Dell'italia LJ, Darley-Usmar VM, Welch DR, Ballinger SW (2013) Mitochondrial genetic background modulates bioenergetics and susceptibility to acute cardiac volume overload. *Biochem J*. **15**, 157-67.

Funk DJ, Omland KE (2003) Species-level paraphyly and polyphyly: frequency, causes and consequences, with insights from animal mitochondrial DNA. *Ann Rev Eco. Evo & Sys.* **34**, 397-423.

Galtier N, Nabholz B, Glémin S, Hurst GDD (2009) Mitochondrial DNA as a marker of molecular diversity: a reappraisal. *Molecular Ecology* **18**, 4541-4550.

Hayati Rezvan P, Lee KJ, Simpson JA (2015) The rise of multiple imputation: a review of the reporting and implementation of the method in medical research. *BMC Med Res Methodol.* **15**, 30.

Hill GE, Havird JC, Sloan DB, Burton RS, Greening C, Dowling DK (2019) Assessing the fitness consequences of mitonuclear interactions in natural populations. *Biol Rev Camb Philos Soc*. **94**, 1089-1104.

Immonen E, Collet M, Goenaga J, Arnqvist G (2016a) Direct and indirect genetic effects of sex-specific mitonuclear epistasis on reproductive ageing. *Heredity* **116**, 338-47.

Immonen E, Rönn J, Watson C, Berger D, Arnqvist G (2016b) Complex mitonuclear interactions and metabolic costs of mating in male seed beetles. *J Evol Biol*. **29**, 360-70.

Innocenti P, Morrow EH, Dowling DK (2011) Experimental evidence supports a sex-specific selective sieve in mitochondrial genome evolution. *Science* **332**, 845-8.

Jakobsen JC, Gluud C, Wetterslev J, Winkel P (2017) When and how should multiple imputation be used for handling missing data in randomised clinical trials - a practical guide with flowcharts. *BMC Med Res Methodol*. **17**, 162.

James AC, Ballard JW (2003) Mitochondrial genotype affects fitness in Drosophila simulans. *Genetics* **164**, 187-94.

James JE, Piganeau G, Eyre-Walker A (2016) The rate of adaptive evolution in animal mitochondria. *Mol Ecol.* **25**, 67-78.

Jumbo-Lucioni P, Bu S, Harbison ST, Slaughter JC, Mackay TFC, Moellering DR, De Luca M (2012) Nuclear genomic control of naturally occurring variation in mitochondrial function in *Drosophila melanogaster*. *BMC Genomics* **13**, 659

Kang H (2013) The prevention and handling of the missing data. *Korean J Anesthesiol.* **64**, 402-6.

Katewa SD, Ballard JW (2007) Sympatric Drosophila simulans flies with distinct mtDNA show difference in mitochondrial respiration and electron transport. *Insect Biochem Mol Biol.* **37**, 213-22.

Keselman HJ (1975) A Monte Carlo investigation of three estimates of treatment magnitude: Epsilon squared, eta squared, and omega squared. *Canadian Psychological Review* **16***,* 44–48.

Koch RE, Dowling DK (2022) Effects of mitochondrial haplotype on pre-copulatory mating success in male fruit flies (*Drosophila melanogaster*). *J Evol Biol* **35**, 1396-1402.

Lajbner Z, Pnini R, Camus MF, Miller J, Dowling DK (2018) Experimental evidence that thermal selection shapes mitochondrial genome evolution. *Sci Rep*. **8**, 9500.

Lamelza P, Ailion M (2017) Cytoplasmic-nuclear incompatibility between wild isolates of *caenorhabditis nouraguensis*. *G3* **7**, 823-834.

Lane N, Martin WF (2010) The energetics of genome complexity. *Nature* **467**, 929–934.

Lane N (2015) *The Vital Question: Energy, Evolution, and the Origins of Complex Life*. W. W. Norton & Company.

Ledesma A, de Lacoba MG, Rial E (2015) The mitochondrial uncoupling proteins. *Genome Biol.* **3**, 3015.

Lee HY, Chou JY, Cheong L, Chang NH, Yang SY, Leu JY (2008) Incompatibility of nuclear and mitochondrial genomes causes hybrid sterility between two yeast species. *Cell* **135**, 1065-1073.

Maklakov AA, Friberg U, Dowling DK, Arnqvist G (2006) Within-population variation in cytoplasmic genes affects female life span and aging in Drosophila melanogaster. *Evolution* **60**, 2081-2086.

Martin WF, Müller M (1998) The hydrogen hypothesis for the first eukaryote. *Nature* **392**, 37–41.

Meiklejohn CD, Montooth KL, Rand DM (2007) Positive and negative selection on the mitochondrial genome. Trends Genet. **23**, 259-263.

Meiklejohn CD, Holmbeck MA, Siddiq MA, Abt DN, Rand DM, Montooth KL (2013) An Incompatibility between a mitochondrial tRNA and its nuclear-encoded tRNA synthetase compromises development and fitness in Drosophila. *PLoS Genet*. **9**, e1003238.

Mishmar D, Ruiz-Pesini E, Golik P, Macaulay V, Clark AG, Hosseini S, Brandon M, Easley K, Chen E, Brown MD, Sukernik RI, Olckers A, Wallace DC (2003) Natural selection shaped regional mtDNA variation in humans. *Proc Natl Acad Sci USA*. **100**, 171-6.

Montooth KL, Meiklejohn CD, Abt DN, Rand DM (2010) Mitochondrial-nuclear epistasis affects fitness within species but does not contribute to fixed incompatibilities between species of Drosophila. *Evolution* **64**, 3364-79.

Morales HE, Pavlova A, Joseph L, Sunnucks P (2015) Positive and purifying selection in mitochondrial genomes of a bird with mitonuclear discordance. *Mol Ecol.* **24**, 2820-37.

Morrow EH, Reinhardt K, Wolff JN, Dowling DK (2015) Risks inherent to mitochondrial replacement. *EMBO Rep*. **16**, 541-4.

Mossman JA, Biancani LM, Zhu CT, Rand DM (2016a) Mitonuclear epistasis for development time and its modification by diet in Drosophila. *Genetics* **203**, 463–484.

Mossman JA, Tross JG, Li N, Wu Z, Rand DM (2016b) Mitochondrial-nuclear interactions mediate sex-specific transcriptional profiles in *Drosophila*. *Genetics* **204** 613–630.

Niehuis O, Judson AK, Gadau J (2008) Cytonuclear genic incompatibilities cause increased mortality in male F2 hybrids of Nasonia giraulti and N. vitripennis. *Genetics* **178**, 413-26.

Osada N, Akashi H (2012) Mitochondrial–nuclear interactions and accelerated compensatory evolution: evidence from the primate cytochrome c oxidase complex. *Mol Biol Evol.* **29**, 337–346.

Pichaud N, Ballard JW, Tanguay RM, Blier PU (2012) Naturally occurring mitochondrial DNA haplotypes exhibit metabolic differences: insight into functional properties of mitochondria. *Evolution* **66**, 3189-3197.

Pichaud N, Ballard JW, Tanguay RM, Blier PU (2013) Mitochondrial haplotype divergences affect specific temperature sensitivity of mitochondrial respiration. *J Bioenerg Biomembr*. **45**, 25-35.

Pozzi A, Dowling DK (2021) Small mitochondrial RNAs as mediators of nuclear gene regulation, and potential implications for human health. *BioEssays* **43**, 2000265.

Rand DM, Clark AG, Kann L (2001) Sexually antagonistic cytonuclear fitness interactions in *Drosophila melanogaster*. *Genetics* **159**, 173–187.

Rand DM, Haney RA, Fry AJ (2004) Cytonclear coevolution: the genomics of cooperation. *Trends in Ecology and Evolution* **19**, 645-653.

Rand DM, Fry A, Sheldahl L (2006) Nuclear-mitochondrial epistasis and drosophila aging: introgression of Drosophila simulans mtDNA modifies longevity in *D. melanogaster* nuclear backgrounds. *Genetics* **172**, 329-41.

Rank NE, Mardulyn P, Heidl SJ, Roberts KT, Zavala NA, Smiley JT, Dahlhoff EP (2020) Mitonuclear mismatch alters performance and reproductive success in naturally-introgressed populations of a montane leaf beetle. *Evolution* **74**, 1724-1740.

Riesenberg L, Soltis D (1991) Phylogenetic consequences of cytoplasmic gene flow in plants. *Evol. Trend Plants* **5**, 65-84

Rowe L, Houle D (1996) The lek paradox and the capture of genetic variance by condition dependent traits. *Proc. R. Soc. Lond. B.* **263**, 1415-1421.

Ruiz-Pesini E, Mishmar D, Brandon M, Procaccio V, Wallace DC (2004) Effects of purifying and adaptive selection on regional variation in human mtDNA. *Science* **303**, 223-226.

Ryan MT, Hoogenraad NJ (2007) Mitochondrial-nuclear communications. *Annual Reviews Biochemistry* **76**, 701-722.

Sackton TB, Haney RA, Rand DM (2003) Cytonuclear coadaptation in Drosophila: disruption of cytochrome c oxidase activity in backcross genotypes. *Evolution* **57**, 2315-25

Sagan L (1967) On the origin of mitosing cells. *Journal of Theoretical Biology* **14**, 225–274

Simard CJ, Pelletier G, Boudreau LH, Hebert-Chatelain E, Pichaud N (2018) Measurement of Mitochondrial Oxygen Consumption in Permeabilized Fibers of *Drosophila* Using Minimal Amounts of Tissue. *J Vis Exp*. **7**, 57376.

Sloan DB, Havird JC, Sharbrough J (2017) The on-again, off-again relationship between mitochondrial genomes and species boundaries. *Mol Ecol.* **26**, 2212-2236.

Spirek M, Polakova S, Jatzova K, Sulo P (2014) Post-zygotic sterility and cytonuclear compatibility limits in S. cerevisiae xenomitochondrial cybrids. *Front Genet*. **5**, 454.

Sterne JA, White IR, Carlin JB, Spratt M, Royston P, Kenward MG, Wood AM, Carpenter JR (2009) Multiple imputation for missing data in epidemiological and clinical research: potential and pitfalls. *BMJ* **29**, 338-393.

Stewart JB, Freyer C, Elson JL, Wredenberg A, Cansu Z, Trifunovic A, Larsson NG (2008) Strong purifying selection in transmission of mammalian mitochondrial DNA. *PLoS Biol.* **6**, e10.

Stewart JB, Larsson NG (2014) Keeping mtDNA in shape between generations. *PLoS Genet*. **10**, e1004670.

Sujkowski A, Spierer AN, Rajagopalan T, Bazzell B, Safdar M, Imsirovic D, Arking R, Rand DM, Wessells R (2019) Mito-nuclear interactions modify Drosophila exercise performance. *Mitochondrion* **47**, 188-205

Taanman JW (1999) The mitochondrial genome: structure, transcription, translation and replication. Biochim Biophys Acta. **1410**, 103-123.

Toews DP, Brelsford A (2012) The biogeography of mitochondrial and nuclear discordance in animals. *Mol Ecol.* **21**, 3907-30.

Troncoso Skidmore S, Thompson B (2013) Bias and precision of some classical ANOVA effect sizes when assumptions are violated. *Behav Res Methods.* **45**, 536-46.

Walker BR, Moraes CT (2022) Nuclear-Mitochondrial Interactions. *Biomolecules* **12**, 427.

Wallace DC, Brown MD, Lott MT (1999) Mitochondrial DNA variation in human evolution and disease. *Gene* **238**, 211-230.

Wang S, Ore MJ, Mikkelsen EK, Lee-Yaw J, Toews DPL, Rohwer S, Irwin D (2021) Signatures of mitonuclear coevolution in a warbler species complex. *Nat Commun*. **12**, 4279.

Wolff JN, Pichaud N, Camus MF, Côté G, Blier PU, Dowling DK (2016) Evolutionary implications of mitochondrial genetic variation: mitochondrial genetic effects on OXPHOS respiration and mitochondrial quantity change with age and sex in fruit flies. *J Evol Biol*. **29**, 736-47.

Wolstenholme DR (1992) Animal mitochondrial DNA: structure and evolution. Int Rev Cytol. **141**, 173-216.

Yee WK, Sutton KL, Dowling DK (2013) In vivo male fertility is affected by naturally occurring mitochondrial haplotypes. *Curr Biol* **23**, R55-6.

Zhang F, Broughton RE (2013) Mitochondrial-nuclear interactions: compensatory evolution or variable functional constraint among vertebrate oxidative phosphorylation genes? *Genome Biol Evol* **5**, 1781-91

Zhu CT, Ingelmo P, Rand DM (2014) G×G×E for lifespan in *Drosophila*: mitochondrial, nuclear, and dietary interactions that modify longevity. *PLoS Genet.* **10**, e1004354.

3.7 | FIGURES

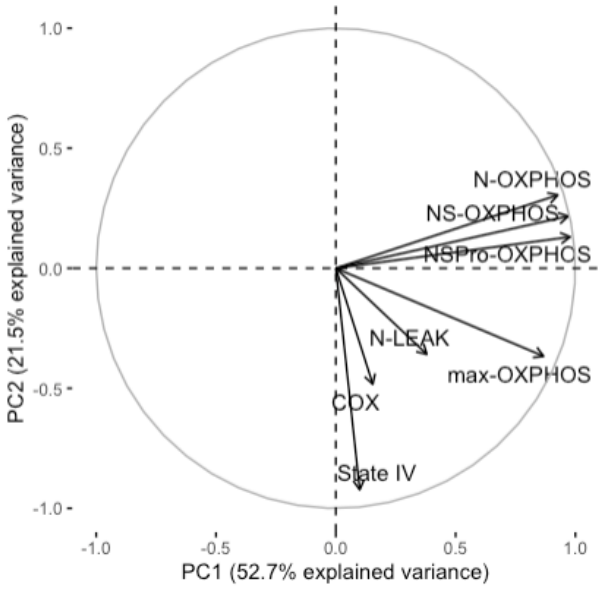

Figure 1. Principal component analysis (PCA) of respiratory parameters across nine mitonuclear strains of *Drosophila melanogaster*. Principal component 1 (PC1) accounted for 52.7% of the variance in the respiratory parameters, with parameters representing coupled respiration (N-OXPHOS, NPro-OXPHOS, NProS-OXPHOS, max-OXPHOS) loading heavily onto this axis. The next principal component (PC2) accounted for 21.5% of the variance and was most strongly associated with parameters that represent uncoupled respiration (through either ADP insufficiency or ATP synthase inhibition - N-LEAK, State IV and COX).

Table 1a. Results of a linear mixed effects model examining the effects of nuclear genome, mitochondrial haplotype and their interaction as well as sex and age on PC1 scores, our primary metric of coupled respiration. Respirometer chamber and block were modelled as random effects. F statistics (calculated with Kenward-Rogers degrees of freedom) are reported along with p-values calculated from a Type III ANOVA. Partial Omega-squared (ω^2) values estimating effect sizes for each term are also reported, along with 95% confidence intervals for their estimates. Estimated SD of random effects and residual values are included.

	PC1				
	Df (num, den)	**F**	**p**	**ω^2 (partial)**	**95% CI**
Nuclear	2, 271.810	3.15*	0.044	0.02	[0.00, 0.05]
Haplotype	2, 271.529	4.52*	0.012	0.03	[0.00, 0.07]
Sex	1, 269.641	44.38***	< 0.001	0.14	[0.07, 0.22]
Age	1, 269.844	40.15***	< 0.001	0.13	[0.06, 0.20]
Nuclear x Haplotype	4, 270.875	0.41	0.800	0.00	[0.00, 0.00]
	SD				
Block	0.75				
Respirometer chamber	0.05				
Residual	1.54				

Table 1b. Results of a linear mixed effects model examining PC2 scores, our proxy of uncoupled (non-ATP producing) respiration, as a response variable. Nuclear genotype, mitochondrial haplotype and their interaction are modelled as fixed effects, along with sex, age, a nuclear x sex x age interaction and all possible pairwise interactions of this 3-way interaction. Respirometer chamber and block were modelled as random effects. F statistics (calculated with Kenward-Rogers degrees of freedom) are reported along with p-values calculated from a Type III ANOVA. Partial Omega-squared (ω^2) values estimating effect sizes for each term are also reported, along with 95% confidence intervals for their estimates. Estimated SD of random effects and residual values are included.

PC2					
	Df (num, den)	**F**	**p**	**ω^2 (partial)**	**95% CI**
Nuclear	2, 264.23	69.10***	< 0.001	0.34	[0.25, 0.42]
Haplotype	2, 264.44	0.16	0.849	0.00	[0.00, 0.00]
Sex	1, 265.00	89.77***	< 0.001	0.25	[0.17, 0.34]
Age	1, 262.39	38.96***	< 0.001	0.13	[0.06, 0.21]
Nuclear x Haplotype	4, 263.17	2.58*	0.038	0.02	[0.00, 0.06]
Nuclear x Sex	2, 264.04	2.63	0.074	0.01	[0.00, 0.05]
Nuclear x Age	2, 264.03	0.46	0.463	0.00	[0.00, 0.00]
Sex x Age	1, 262.63	3.03	0.082	0.007	[0.00, 0.04]
Nuclear x Sex x Age	2, 264.40	3.73*	0.025	0.02	[0.00, 0.06]
	SD				
Block	0.61				

Respirometer chamber	0.09
Residual	0.75

Table 1c. Results of a linear mixed effects model examining respiratory control ratio (RCR) scores, our proxy of respiratory efficiency, as a response variable. Nuclear genotype, mitochondrial haplotype and their interaction are modelled as fixed effects, along with sex, age, a nuclear x sex x age interaction and all possible pairwise interactions of this 3-way interaction. Respirometer chamber and block were modelled as random effects. F statistics (calculated with Kenward-Rogers degrees of freedom) are reported along with p-values calculated from a Type III ANOVA. Partial Omega-squared (ω^2) values estimating effect sizes for each term are also reported, along with 95% confidence intervals for their estimates. Estimated SD of random effects and residual values are included.

Respiratory Control Ratio					
	Df (num, den)	**F**	**p**	**ω^2 (partial)**	**95% CI**
Nuclear	2, 266.91	57.82***	< 0.001	0.30	[0.21, 0.38]
Haplotype	2, 266.60	1.77	0.172	0.006	[0.00, 0.03]
Sex	1, 267.00	209.98***	< 0.001	0.44	[0.36, 0.51]
Age	1, 264.90	136.43***	< 0.001	0.34	[0.25, 0.42]
Nuclear x Haplotype	4, 265.40	0.51	0.730	0.00	[0.00, 0.00]
Nuclear x Sex	2, 266.03	1.94	0.145	0.007	[0.00, 0.03]
Nuclear x Age	2, 266.37	5.13**	0.007	0.03	[0.00, 0.08]
Sex x Age	1, 264.90	26.22***	<0.001	0.09	[0.03, 0.16]

	SD
Block	0.16
Respirometer chamber	0.05
Residual	0.42

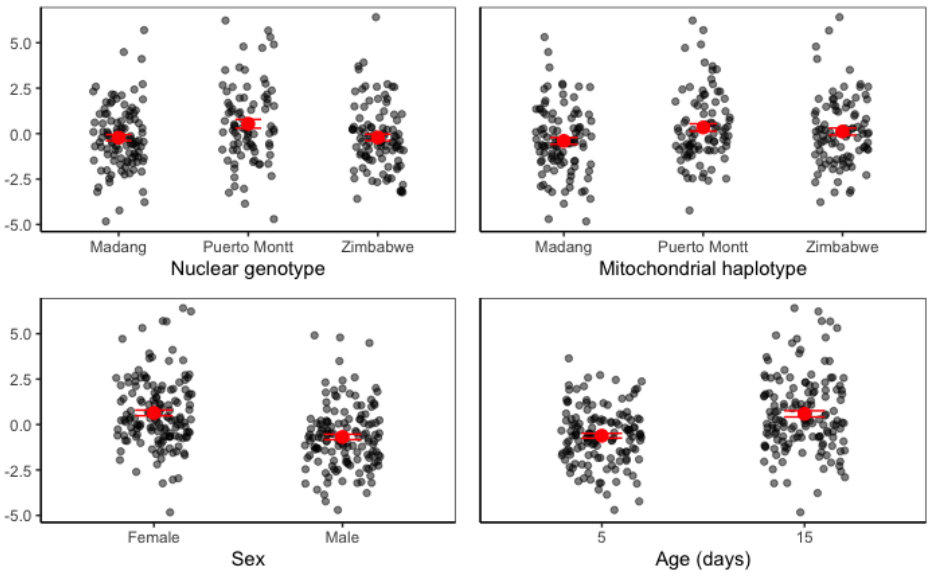

Figure 2. Effects of nuclear genotype (a), mitochondrial haplotype (b), sex (c) and age (d) on PC1 scores; our primary metric of coupled respiration. Black dots represent individual samples, with red dots and bars signalling means and standard error respectively. Each variable significantly influenced PC1 scores.

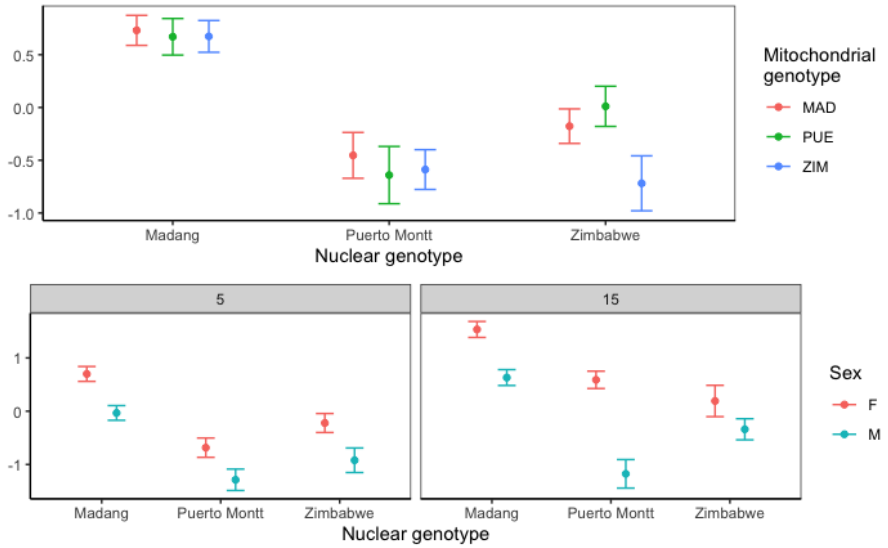

Figure 3. Combined effects of nuclear genotype and mitochondrial haplotype (a), along with effects of nuclear genotype, sex and age (b,c) on PC2 scores; our metric for uncoupled respiration. Dots represent sample means with standard error bars attached. Plot (a) displays a significant mtDNA haplotype x nuclear genotype interaction, and plots (b) and (c) together display a significant three-way interaction between nuclear genotype, age and sex.

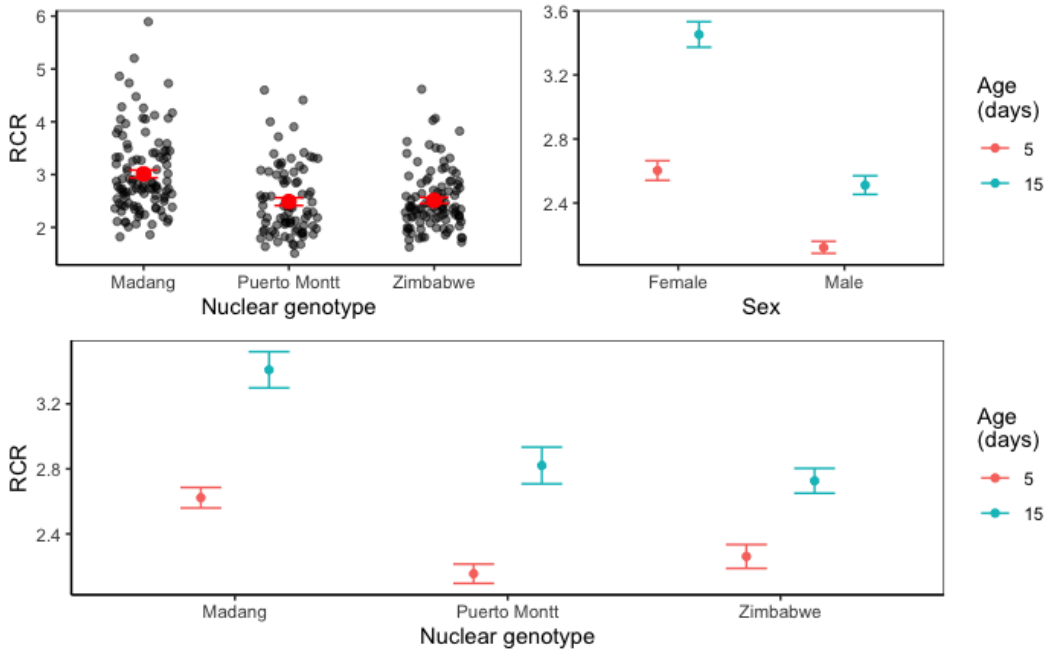

Figure 4. Effects of nuclear genotype (a), and the interactions between nuclear genotype x sex (b) and age x sex (c) on respiratory control ratio; our metric of bioenergetic efficiency. Age and sex both

produced significant main effects that are not explicitly plotted here. Black dots represent individual RCR values, and red dots represent sample means with standard error bars attached.

CHAPTER IV | Sexual selection shapes the evolution of respiratory control ratio in *Drosophila melanogaster*

Tom M. Allison, Winston K.W. Yee, Craig R. White, Damian K.Dowling

4.1 | ABSTRACT

Theory predicts that, in sexually reproducing organisms, those with a greater supply of usable energy are more likely to secure a mate. This may be achieved through increased allocation to traits related to competition for mates, such as fighting, posturing, maintaining energetically expensive ornaments or costly displays of endurance. We assessed whether populations of *Drosophila melanogaster* experimentally evolved under either high or low sexual selection pressures showed differences in electron transport system (ETS) function. We found that the strength of sexual selection does not influence the maximum possible throughput of the ETS, but it does influence the stoichiometric *efficiency* of respiration; that is, the amount of ATP produced per fuel source and oxygen. Organisms subjected to high sexual selection showed increased respiratory efficiency, suggesting that intense sexual selection favours tighter coupling, despite potential costs due to increased production of reactive oxygen species. In the context of other work linking intense sexual selection with a faster 'pace-of-life', our findings suggest that respiratory efficiency may act as a mechanism by which the trade-off between allocation to sexually selected traits and somatic maintenance is mediated.

4.2 | INTRODUCTION

The ability to capture the energy from electron transfer reactions and channel it into readily usable forms of energy is a core function of life on earth (Smith & Morowitz 2016). In eukaryotes, this task is accomplished by using free energy released when electrons are passed through successively lower energy states to power a 'proton gradient' across a thin membrane in mitochondria, which is then dissipated through a membrane-bound protein (ATP synthase) to produce ATP (Mitchell 1961). Both the volume and efficiency with which organisms can complete this task have likely been under strong and persistent natural selection for as long as eukaryotes have existed (Lane & Martin 2010). However, natural selection is not the only criterion by which organismal traits are selected, and the strength and direction of different types of selection on organismal phenotypes do not always align (Arnqvist & Rowe 2005). An important dimension of selection in sexually reproducing metazoans is sexual selection; that is, selection on traits related to competition for fertilisation of gametes, including male-male competition and 'female choice' (Andersson 1994). The traits most strongly favoured under sexual selection may be in concordance, in conflict, or neutral relative to traits and trait values favoured under natural selection (Arnqvist & Rowe 2005; Cally *et al*. 2019).

The range of genes subject to sexual selection is thought to be large, since sexually selected traits often depend on overall 'condition'; a complex, multivariate trait with a hierarchy of contributing factors (Rowe & Houle 1996; Hunt *et al*. 2004; Cotton *et al*. 2004). A common conceptual model places 'subordinate' traits such as metabolic capacity at the base of this hierarchy (Rowe & Houle 1996; Hill 2011; Storz *et al*. 2015) and thus an increase in capacity to produce energy is thought to allow for increased expression of a wide range of condition-

dependent, sexually selected traits including energetically expensive courtship displays, costly morphological features and gamete production (Watson *et al*. 1998; Hunt *et al*. 2004; Somjee *et al*. 2018). In accordance, researchers have recently demonstrated that the fruit fly *Drosophila pseudoobscura* evolves higher metabolic rates when subjected to experimentally enforced polyandry, compared to experimentally enforced monogamy (Garlovsky *et al*. 2022). While metabolic rate may evolve in response to sexual selection, the mechanisms moderating these changes are not well known.

The electron transport system that supports oxidative phosphorylation is comprised of several large protein 'complexes' that ferry electrons from one to the next and use the energy released to transport hydrogen ions across a phospholipid membrane. One mechanism by which a higher metabolic rate could be supported is through increased flux of electrons through individual protein complexes of the electron transport system. However, this is not the only means by which increased metabolic demands may be met. Plastic responses to increased energetic demands include changes to vasculature (Clausen 1977; Green *et al*. 2017), density of oxygen carrying molecules (Mairbaurl 1994; Hochachka *et al*. 1996), increases in mitochondrial density via mitochondrial biogenesis (Gutsaeva *et al*. 2008; Jornayvaz & Shulman 2010), changes to mitochondrial mitochondrial membrane morphology (Cogliati *et al*. 2016) and finally mitochondrial network dynamics (Liesa & Shirihai 2013). In the case of increased metabolic capacity associated with heightened sexual selection, it remains to be seen whether increased electron flux through the ETS is one of the physiological adaptations that support a higher metabolic rate.

Increasing flux of electrons through the electron transport system requires not only adjustments to the ETS to support the flux, but also to organismal resource acquisition. Another way to increase metabolic capacity without necessarily increasing resource acquisition is by increasing the efficiency with which oxygen and organic reducing equivalents are converted to usable forms of energy (ATP). Although there is no universally agreed upon metric for bioenergetic efficiency, it has been argued that of all respiratory parameters, the respiratory control ratio (RCR) is the most relevant (Brand & Esteves 2005; Brand & Nicholls 2011; Koch *et al*. 2021). This ratio describes the ratio of oxygen that is consumed when ATP synthase is functioning compared to when it is inhibited. The oxygen consumption when ATP synthase is inhibited, termed LEAK or State IV respiration (depending on presence/abundance of ADP and Krebs cycle intermediates), is attributable to maintenance of the proton gradient as hydrogen ions 'slip' across the mitochondrial inner membrane. If LEAK or State IV oxygen consumption is high relative to maximum oxygen consumption during oxidative phosphorylation, it means that the inner mitochondrial membrane may be 'leaky' – or wasteful – of hydrogen ions and may indicate 'inefficient' respiration (Brand & Esteves 2005; Cheng et al. 2017).

There are various means by which sexual selection could influence bioenergetic parameters, including ETS electron flux and measures of efficiency such as the respiratory control ratio. In a pre-copulatory context, intense male-male competition demands increased metabolic rate, which may or may not be associated with increased electron flux through individual complexes of the ETS. Independent of ETS electron flux, higher metabolic demands in association with finite caloric intake may demand increases in OxPhos efficiency. Regarding female choice, it has been argued that the ability to efficiently process resources through

the electron transport system constitutes a primary criterion upon which organisms may select a mate (Hill 2011, Hill *et al*. 2019). As such, honest signals representing such function are expected to evolve. Indeed, research has demonstrated that colouration – a trait known to be under selection through female choice – in male house finches is linked to mitochondrial coupling efficiency.

Even in the domain of cryptic female choice (sperm competition within the reproductive tract of 'promiscuous' females), there may be room for selection on bioenergetic parameters. This is because sperm traits such as quantity, swimming speed and endurance, capacity to cope with chemical barriers, and ability to penetrate the egg faster than competitors dictate fertilisation success (Snook 2005; Pizzari & Parker 2009; Parker 2020). Each of these traits may rely on energetic input from mitochondria within the sperm itself, or, perhaps more commonly from the somatic tissue of the male. Indeed, production of the ejaculate (including sperm and additional nutritive substances) is thought to carry substantial energetic costs in many species (Dewsbury 1982; Olsson *et al*. 1997; Immonen *et al*. 2016). While the literature suggests that links exist between metabolic capacity – either as increased electron flux through the ETS or an increase in respiratory efficiency – may contribute toward performance in sexually selected traits, no study has yet attempted to directly assess capacity for evolutionary change in bioenergetic parameters under variation in the strength of sexual selection.

Here, we test whether differences in levels of sexual selection applied to populations of the fruit fly *D. melanogaster* are associated with changes in key bioenergetic parameters, and whether these differences vary between males and females. We utilise an experimental

evolutionary design that retains selection (both sexual and natural selection) on both sexes, and which varies only in the magnitude and opportunity for sexual selection to occur. This allowed us to remain agnostic to whether there is concordance or conflict between the sexes and ask whether stronger sexual selection per se impacts bioenergetic function over generations.

4.3 | MATERIALS & METHODS

4.3.1 | Experimental evolution populations

Experimentally evolved populations were maintained under their respective selection treatments from 2008 to present (approx ~350 generations) and maintenance methods have been described in detail elsewhere (Innocenti *et al*. 2014, Nystrand *et al*. 2018). Briefly, founding flies were sourced from a large, panmictic population (LH_M) (Rice *et al*. 2006). 384 virgin female LH_M and 384 virgin male LH_M flies were collected and randomly allocated to one of eight replicate populations, which were then each assigned to a mating treatment of either low or high sexual selection. Mating treatments differed only in the duration that adult male and female flies were permitted contact (though this primary asymmetry has several secondary consequences for mating structure that will be discussed below). Each generation, several vials per replicate population were populated with 16 pairs of three-day-old virgin flies, which were subsequently allowed to mate. In the low sexual selection treatment, flies were only permitted a total of one hour to mate before males were removed (and females left in the vials for a further 23 hours). In the high sexual selection treatment, males remained in the vials with females for 24 hours. In each treatment, at the

24 hour mark, females were transferred to fresh vials and allowed to oviposit for the next 18 hours. In the high sexual selection treatment, male flies continued to cohabit with females during this 18-hour laying window. In each treatment, the eggs which go on to form the next generation of respective populations, were trimmed to 120 per vial. All experimental populations were kept under standard rearing conditions of 25°C, 12 L:12D, and 60% humidity, except for an infrastructure malfunction (approx. one year/25 generations before the current experiment) that caused a temporary spike in housing temperature to ~40 °C for several hours. While this malfunction placed all populations under selection for extreme heat tolerance, we note that all replicate populations survived this heat stress without a reduction in the number of offspring contributing to the next generation.

The differences between the two selection treatments ensure that the strength of sexual selection is different across several dimensions of sexual selection. According to careful observation, the mating refractory period of females in the lineages used in the present study is such that a one-hour mating window permits enough time for only a single mating in almost all cases (Innocenti *et al*. 2014, Kuijper & Morrow 2009). Previous data on these populations have confirmed that females start to remate after approximately 2 hours when kept under *en masse* conditions (Innocenti et al 2014). Thus, the low sexual selection treatment effectively enforces monogamy and eliminates the potential for post-copulatory forms of sexual selection. Data from other populations of *D. melanogaster* show that 80%–100% of young females (3–4 days old) will remate within 20 hours of first mating (Fricke et al. 2014). It is known that male *D. melanogaster* will vigorously court females during the post-copulatory refractory period, and thus, a longer interval of contact between the sexes

gives females ample opportunity to “trade-up” by remating with other males. We contend, therefore, that the total time with which males and females remain in contact (one hour in the low-sexual selection versus 42 hours in the high-sexual selection treatment) is likely to foster quantitative differences in pre-copulatory forms of sexual selection and qualitative differences in post-copulatory forms of sexual selection. We note that since females in wild populations of *D. melanogaster* mate multiply, it is the low sexual selection treatment that deviates most from the natural mating system, and thus serves as the ‘treatment’, whereas the high sexual selection system serves as an approximate ‘control’.

Each replicate population was initially maintained at a population size of 96 (three vials per replicate, each containing 16 pairs), which were admixed as adults each generation, and then redistributed back into three vials). However, after ~95 generations of experimental evolution, population size was increased to 224 (seven vials per replicate, each containing 16 pairs). Within-population panmixia was ensured via admixture of eclosing virgin flies from each of the seven vials and subsequent random sorting into sex-specific vials (where they were held before pairing at three days of age) at each generation. A second change made at this point was a pivot from cornmeal-molasses-yeast-agar medium to a potato-dextrose-agar medium (37.32% yeast, 31.91% dextrose, 23.40% potato, and 7.45% agar combined with 98.48% H_2O, 0.97% ethanol, 0.45% propionic acid, and 0.11% nipagen).

4.3.2 | Fruit-fly preparation and Common Garden

Flies of each sex and population were sampled at ten days of age (post eclosion). Since assays were conducted over several consecutive days, each requiring adult flies that were exactly 10 days of adult age, we initiated a breeding strategy that ensured a continuous

production of focal flies across successive days. To achieve this, we ensured that the grandparental flies of the focal flies used in the experiment mated and laid eggs over successive days. For each of the eight replicate populations, 32 pairs of adult (grand-parental) flies were collected and allocated across two vials. Beginning at 3 days of age, flies were allowed to mate and oviposit for 24 hours in 40mL vials containing 6mL food media (as described above) and excess eggs were trimmed to approximately 100. Adult pairs from each strain were transferred into fresh vials after each 24 hour period (hereafter termed a 'lay'), for a total of ten consecutive days. The result was a set of ten staggered 'parent' groups (parents to the focal flies) that eclosed from each of these vials, differing from one to the next by only the age of their parents at the time of ovipositioning. Parents produced from each lay were collected at five days-of-age, maintained across two vials (again with 16 pairs per vial), and allowed to cohabit, mate and oviposit for 24 hours. Eggs produced over this 24 h lay were trimmed to approximately 100. From the resulting offspring, 24 virgin flies per sex were collected from each of the eight populations and maintained across four vials (6 per vial) to minimise density effects. The net result was a set of ten staggered groups of focal flies, separated only by the age of their grandparents (with a maximum of ten days difference between the first and last lay). Focal flies were transferred to new vials every three days until sampling. In addition to standardising parental effects and focal fly age at assay, this procedure provided a 'common garden' environment for all populations, where low sexual selection was relaxed for two generations.

4.3.3 | High resolution respirometry measurements

For measurement of fruit-fly respiration, we followed methods described in Simard et al (2018). Briefly, thorax muscle (3 thoraces per sample for male flies, and 2 thoraces per

sample for female flies) were permeabilized at 4 °C using BIOPS relaxing solution (2.77mM CaK_2EGTA, 7.23mM K_2EGTA, 5.77 mM Na_2ATP, 6.56mM $MgCl_2$, 20mM taurine, 15mM Na_2phosphocreatine, 20mM imidazole, 0.5mM dithiothreitol, 50mM K-MES, pH 7.2) complemented with 62.5µg/mL saponin, and subsequently blot-dried, weighed and transferred into the respiration chamber of an Oxygraph-2k respirometer (Oroboros Instruments, Innsbruck, Austria), calibrated with air-saturated respiration medium (115 mM KCl, 10 mM KH_2PO_4, 2 mM $MgCl_2$, 3 mM HEPES, 1 mM EGTA, 0.2% BSA, pH 7.2) at 25 °C. Prior to the transfer of fibres, pyruvate and malate (10 mM each) were added to the respiration medium in order to stimulate the NADH-pathway without oxidative phosphorylation (N-LEAK). After monitoring for stabilisation of N-LEAK, we added ADP (5 mM) to measure oxidative phosphorylation sustained only by complex I substrates (N-OXPHOS). Next, we assessed the integrity of the outer mitochondrial membrane through addition of cytochrome c (15 µM), with samples showing a rise of ≥15% in oxygen consumption discarded. We then added sequential injections of the following substrates: succinate (10 mM) to stimulate complex II (NS-OXPHOS), proline (10 mM) to fuel proline dehydrogenase (NSPro-OXPHOS), and *sn* glycerol-3-phosphate (20 mM) to stimulate glycerol-3-phosphate dehydrogenase and induce maximum oxidative phosphorylation (max-OXPHOS). After measurement of max-OXPHOS, oligomycin (0.01 µM) was added to inhibit the function of ATP synthase (State 4). Our calculation for the Respiratory Control Ratio (RCR) was simply max-OXPHOS divided by State 4 respiration (Brand & Nicholls 2011).

Once the State 4 signal had levelled, rotenone (1 µM), malonate (10 mM) and antimycin A (2.5 µM) were added to inhibit complex I, complex II and complex III respectively, allowing for measurement of residual oxygen consumption. Finally, N,N,N',N'-tetramethyl-p-

phenylenediamine (TMPD, 0.5 µM) and ascorbate (2 mM) were added to measure complex IV activity (COX), which was corrected for chemical background oxidation after complete inhibition of COX by sodium azide (20 mM).

All measurements are expressed as means of respiration rates in picomol of oxygen consumed per second and per mg of permeabilized fibres (pmol s^{-1} mg^{-1}). Data was collected for a total of 144 samples, spread evenly across the eight strains and both sexes.

4.3.4 | Statistical Analysis

All analyses were conducted using R software (4.2.0).

Raw oxygen consumption scores showed high correlations across various states of ETS stimulation. Accordingly, we reduced the dimensionality of the dataset using a principal component analysis. Principal component scores were modelled separately to RCR ratio data since the ratio was both of specific biological interest à priori and was not strongly correlated with any of the other bioenergetic parameters in the dataset.

Raw data for RCR ratio was significantly non-normal under Shapiro-Wilks test ($W = 0.92$, $p < 0.001$) and a range of transformations (log, square-root, and box-cox) were not sufficient to normalise the data. However, visualisation of simulation of scaled residuals for the fitted linear mixed model showed no significant deviation from uniformity. This was supported statistically with a non-significant KS-test ($p = 0.57$) and a non-significant Dispersion test ($p = 0.73$). These findings suggest that the fitted linear mixed model is likely robust to non-normality (Schielzeth *et al*. 2020). Indeed, removal of data points indicated to be influential

by cook's distance allowed for normality of the data but did not alter the significance of any fixed terms in the model. As such, we proceeded with fitting linear models to the complete set of raw RCR values.

PC1 and PC2, and separately, RCR values were fit to general linear mixed models, with mating treatment, sex and their interaction included as fixed effects. Random effects included respirometer chamber (four distinct chambers were used in sampling) and the interaction between replicate and sex. This random interaction term was chosen to represent the possibility that sex effects on each parameter varied across population replicates, whilst also maintaining an appropriate denominator degrees of freedom for sex and treatment main effects (and their interaction) that represented the true level of replication in the study design (the number of replicates, n = 8) (Arnqvist 2020). Modelling an interactive random effect was preferable over modelling sex as a random slope within each replicate, since the latter failed to accurately estimate the correlation between sex and line, thus precipitating a model boundary fit (singularity) error. Residual maximum likelihood was used to estimate the variance of the model parameters. F-tests with Kenward-Rogers approximation of degrees of freedom, and a type III model with sum-to-zero contrasts were used to assess the significance of fixed terms.

4.4 | RESULTS

The primary metric of respiratory efficiency – the respiratory control ratio – responded to the sexual selection treatment. Specifically, flies sampled from populations subjected to the low sexual selection treatment showed reduced respiratory control ratios in comparison to

their high-sexual selection counterparts ($F_{1,12} = 6.35$, $p = 0.027$). Furthermore, females showed consistently higher RCR scores than males across both selection treatments, reflected in a significant main effect of sex ($F_{1,12} = 5.99$, $p = 0.031$) and a non-significant interaction between sex and mating treatment ($F_{1,12} = 0.29$, $p = 0.599$).

Under principal component analysis, respiratory parameters related to coupled (OXPHOS) respiration, such as N-OXPHOS, NPro-OXPHOS, NProS-OXPHOS, max-OXPHOS), explained most of the overall variance in our dataset. Each of these parameters loaded heavily onto principal component one (PC1), which accounted for 64.8% of variation among respiratory parameter scores (Fig 2). Principal component two (PC2) explained 20.9% of variation and was best represented by respiratory parameters related to uncoupled respiration, such as N-LEAK (uncoupled through ADP insufficiency) and State IV (uncoupled through inhibition of ATP synthase).

There were no effects of treatment or sex on either PC1, the dimension of variation representing coupled respiration ($F_{1,13} = 0.22$, $p = 0.646$ and $F_{1,13} = 2.77$, $p = 0.120$ respectively), or PC2, the dimension representing uncoupled respiration ($F_{1,13} = 2.61$, $p = 0.130$ and $F_{1,13} = 4.07$, $p = 0.065$ respectively).

4.5 | DISCUSSION

We demonstrate that a key measure of respiratory efficiency, the respiratory control ratio, can evolve under experimental manipulation of sexual selection strength. Respiratory control ratios were consistently lower in flies from populations that had evolved under

experimentally enforced low sexual selection, indicating that these populations have evolved lower efficiency in converting oxygen and organic inputs into ATP. Both sexes responded in the same direction across mating treatments, yet female flies demonstrated consistently higher RCR ratios than male flies across both treatments. Our findings build upon prior research showing that a reduction in the strength of sexual selection can lead to the evolution of decreased metabolic rate in both sexes (Garlovsky *et al*. 2022), by showing that reduced metabolic efficiency may similarly evolve when sexual selection is relaxed. In other words, increases in metabolic rate and efficiency appear to be adaptations to heightened sexual selection; not only are organisms under heightened sexual selection consuming more oxygen, but they are producing more ATP *per oxygen* consumed.

The present study represents the first demonstration that evolutionary changes in RCR are associated with changes in the strength of sexual selection. It implies a functional importance for mitochondrial coupling efficiency beyond its role in thermoregulation, for which it is thought to be under natural selection (at least in endotherms) (Enerback *et al*. 1997; Nicholls & Locke 1984; Mishmar *et al*. 2003; Nowack *et al*. 2017; Bertholet & Kirichok 2022). Indeed, earlier work on the on bioenergetic coupling efficiency has suggested a mechanistic role for RCR in mediating life history trade-offs (Brand 2000; Speakman *et al*. 2004). The 'uncoupling to survive' hypothesis suggests that while tighter respiratory coupling (higher RCR ratio) can increase ATP production efficiency, it can incidentally increase production of harmful reactive oxygen species, which may shorten lifespan (Brand 2000). Separately, other research has shown that populations that evolve under higher levels of sexual selection tend to develop and reproduce with increased urgency in a 'live fast, die young' phenotype (Nandy *et al*. 2013; Tarka *et al*. 2018), though the effects may be

sex-specific (Hämäläinen *et al*. 2018; Immonen *et al*. 2018). Thus, it is an intriguing possibility that the increased RCR in flies exposed to heightened sexual selection in the present study demonstrates one mechanism by which organisms can increase performance in some life-history components, but possibly at the cost of performance in others. In this model, increased ATP availability for a given amount of calorie intake would allow greater allocation of energy to costly competitive behaviours related to sexual selection, though this may come at the cost of increased ROS production and shortened lifespan (Dowling & Simmons 2009). Such a possibility requires further research attention.

Whether the high RCR phenotype observed in flies exposed to high sexual selection pressures is adaptive in both sexes or in only one sex is an open question. Intralocus sexual conflict over metabolic related traits has been observed before. For example, in seed beetles (*Calosubruchus maculatus*), male-limited sexual selection drives an increase in whole animal metabolic rate in both sexes despite reducing fitness in females (Berger *et al*. 2014). Genes controlling respiratory efficiency are likely shared across the sexes and there is evidence that optimal RCR values are different in each sex (Chapter III). Shared genetic architecture in a trait with sex-specific optima sets the stage for intralocus sexual conflict (Trivers 1972; Parker 1979; Arnqvist & Rowe 2005; Bonduriansky & Chenoweth 2009). However, a previous study using the same experimentally evolved populations used in the present study showed that females in the high sexual selection treatment had comparatively greater fitness than females in the low sexual selection treatment (Innocenti *et al*. 2014). Thus, the increase in RCR observed in the present study is less likely to be detrimental to females. Furthermore, a subsequent study using the same experimental populations showed that males exposed to the high sexual selection treatment had higher

fitness when exposed to an immune challenge (Nystrand *et al*. 2018), rendering it unlikely that the increase in RCR is detrimental to males. It appears, therefore, that evidence supports mutual benefits, rather than intralocus sexual conflict over RCR values.

While we have demonstrated that coupling efficiency shifts under changes in intensity of sexual selection, the precise selective force driving the changes cannot be resolved from the present study. Some candidate dynamics include male vs male competition, female choice, protective responses in females to high levels of harassment by males, and any of the various forms of post-copulatory sexual selection. Increases in coupling efficiency could plausibly affect expression of each of these processes and elucidating their relative importance as selective forces will require assessment of correlations between RCR and each trait in question. Of particular interest is understanding the links between coupling efficiency and intersexual displays. In many species, courtship displays are energetically expensive, and consequently they may present as honest signals of mitochondrial function (Hill 2011; Hill 2018). In *D. melanogaster*, for example, displays include a choreographed sequence of acoustic, chemical and sensorimotor displays that are thought to depend sensitively on ATP production. Previous work on house finches has already linked RCR with colouration – a trait known to determine attractiveness (Hill *et al*. 2019). The present study, together with previous work on birds, suggests that mitochondrial function may contribute to signals of genetic quality. We contend that establishing further associations between coupling efficiency and various components of fitness (pre- and post-copulatory reproductive success in males and lifetime fecundity in females).

In assessing the validity and robustness of our inferences linking changes in RCR to changes in sexual selection strength, we must address the potential confound between altering the strength of sexual selection and the consequent effect on the effective population sizes (Ne) of our treatment groups. Indeed, as the strength of sexual selection increases, variance of male reproductive success is likely to increase, and this may reduce Ne. Thus, populations placed under high sexual selection may be more prone to evolution by drift than those under low sexual selection. Notwithstanding, we note two key points. Firstly, previous research has demonstrated that drift likely does not play a strong role in shaping evolution of populations of *Drosophila* species placed under similar selection regimes that we applied in our study. Rice and Holland (2005) and Snook *et al*. (2009) assessed populations of *D. melanogaster* and *D. pseudoobscura* respectively, using both census data and molecular markers to estimate Ne in populations that had been exposed to male-limited sexual selection. They conclude that neither Ne, nor genetic diversity was significantly different between treatment groups, indicating that drift is unlikely influential in shaping differences between the experimental populations evolving under each treatment. Secondly, we note that our study addressed directional changes in a metabolic trait. Consistent directional changes across our four replicate populations per treatment are much more likely indicative of selection than drift, the latter of which would likely produce random bi-directional changes.

In summary, our work demonstrates, for the first time, the propensity for an important metric of bioenergetic efficiency to evolve under divergent sexual selection. Our work implies that organisms subjected to heightened sexual selection will evolve higher respiratory efficiency. We suggest that, although high RCR ratios imply increased efficiency,

the optimal level of respiratory efficiency will depend on ecological parameters, one of which is the intensity of sexual selection. Given physiological associations with high RCR and harmful ROS production, RCR rates may be subject to life-history trade-offs in a context-specific manner. Our work stands as a proof-of-concept of the evolvability of bioenergetic parameters under sexual selection and raises important questions about the adaptiveness of high RCR in both sexes and the precise forms of sexual selection that are responsible for effecting the changes observed.

4.6 | REFERENCES

Andersson M (1994) *Sexual Selection*. Princeton University Press, Princeton, NJ.

Arnqvist G, Rowe L (2005) *Sexual Conflict*. Princeton University Press.

Arnqvist G (2020) Mixed Models Offer No Freedom from Degrees of Freedom. *Trends Ecol Evol.* **35**, 329-335.

Berger D, Berg EC, Widegren W, Arnqvist G, Maklakov AA (2014) Multivariate intralocus sexual conflict in seed beetles. *Evolution* **68**, 3457-69.

Bertholet AM, Kirichok Y (2022) Mitochondrial H^+ Leak and Thermogenesis. *Annu Rev Physiol*. **84**, 381-407.

Bonduriansky R, Chenoweth SF (2009) Intralocus sexual conflict. *Trends Ecol Evol.* **24**, 280-8.

Brand MD (2000) Uncoupling to survive? The role of mitochondrial inefficiency in ageing. *Exp Gerontol.* **35**, 811-20.

Brand MD, Esteves TC (2005) Physiological functions of the mitochondrial uncoupling proteins UCP2 and UCP3. *Cell Metab.* **2**, 85-93.

Brand MD, Nicholls DG (2011) Assessing mitochondrial dysfunction in cells. *Biochem J.* **435**, 297-312.

Cally JG, Stuart-Fox D, Holman L (2019) Meta-analytic evidence that sexual selection improves population fitness. *Nat Commun*. **10**, 2017.

Cheng J, Nanayakkara G, Shao Y, Cueto R, Wang L, Yang WY, Tian Y, Wang H, Yang X (2017) Mitochondrial Proton Leak Plays a Critical Role in Pathogenesis of Cardiovascular Diseases. *Adv Exp Med Biol.* **982**, 359-370.

Cogliati S, Enriquez JA, Scorrano L (2016) Mitochondrial Cristae: Where Beauty Meets Functionality. *Trends Biochem Sci* **41**, 261-273.

Dowling DK, Simmons LW (2009) Reactive oxygen species as universal constraints in life-history evolution. *Proc. R. Soc. B.* **276**, 1737-1745.

Enerbäck S, Jacobsson A, Simpson EM, Guerra C, Yamashita H, Harper ME, Kozak LP (1997) Mice lacking mitochondrial uncoupling protein are cold-sensitive but not obese. *Nature* **387**, 90-4.

Fricke C, Green D, Smith D, Dalmay T, Chapman T (2014) MicroRNAs influence reproductive responses by females to male sex peptide in *Drosophila melanogaster*. *Genetics* **198**, 1603-19.

Garlovsky MD, Holman L, Brooks AL, Novicic ZK, Snook RR (2022) Experimental sexual selection affects the evolution of physiological and life-history traits. *J Evol Biol* **35**, 742-751.

Gutsaeva DR, Carraway MS, Suliman HB, Demchenko IT, Shitara H, Yonekawa H, Piantadosi CA (2008) Transient hypoxia stimulates mitochondrial biogenesis in brain subcortex by a neuronal nitric oxide synthase-dependent mechanism. *J Neurosci* **28**, 2015-24

Hämäläinen A, Immonen E, Tarka M, Shuett W (2018) Evolution of sex-specific pace-of-life syndromes: causes and consequences. *Behav. Ecol. Sociobiol.* **72**, 50

Hill GE (2011) Condition-dependent traits as signals of the functionality of vital cellular processes. *Ecol Lett* **14**, 625-34.

Hill GE (2014) Mitonuclear Mate Choice: A Missing Component of Sexual Selection Theory? *Bioessays* **40**

Hill GE, Hood WR, Ge Z, Grinter R, Greening C, Johnson JD, Park NR, Taylor HA, Andreasen VA, Powers MJ, Justyn NM, Parry HA, Kavazis AN, Zhang Y (2019) Plumage redness signals mitochondrial function in the house finch. *Proc Biol Sci.* **286**, 20191354.

Hochachka PW, Buck LT, Doll CJ, Land SC (1996) Unifying theory of hypoxia tolerance: molecular/metabolic defense and rescue mechanisms for surviving oxygen lack. *Proc Natl Acad Sci USA* **93**, 9493-8.

Hunt J, Brooks R, Jennions MD, Smith MJ, Bentsen CL, Bussière LF (2004) High-quality male field crickets invest heavily in sexual display but die young. *Nature* **432**, 1024-7.

Immonen E, Rönn J, Watson C, Berger D, Arnqvist G (2016) Complex mitonuclear interactions and metabolic costs of mating in male seed beetles. *J Evol Biol*. **29**, 360-70.

Immonen E, Hämäläinen A, Schuett W, Tarka M (2018) Evolution of sex-specific pace-of-life syndromes: genetic architecture and physiological mechanisms. *Behav Ecol Sociobiol.* **72**, 60.

Innocenti P, Flis I, Morrow EH (2014) Female responses to experimental removal of sexual selection components in Drosophila melanogaster. *BMC Evol Biol*. **14**, 239.

Jornayvaz FR, Shulman GI (2010) Regulation of mitochondrial biogenesis. Essays Biochem **47**, 69-84

Kienzle L, Bettinazzi S, Choquette T, Brunet M, Khorami HH, Jacques JF, Moreau M, Roucou X, Landry CR, Angers A, Breton S (2023) A small protein coded within the mitochondrial canonical gene nd4 regulates mitochondrial bioenergetics. BMC Biol. **21**, 111.

Koch RE, Buchanan KL, Casagrande S, Crino O, Dowling DK, Hill GE, Hood WR, McKenzie M, Mariette MM, Noble DWA, Pavlova A, Seebacher F, Sunnucks P, Udino E, White CR, Salin K, Stier A (2021) Integrating Mitochondrial Aerobic Metabolism into Ecology and Evolution. *Trends Ecol Evol*. **36**, 321-332.

Kuijper B, Morrow EH (2009) Direct observation of female mating frequency using time-lapse photography. *Fly* **3**, 118-20.

Lane N, Martin WF (2010) The energetics of genome complexity. *Nature* **467**, 929–934.

Liesa M, Shirihai OS (2013) Mitochondrial dynamics in the regulation of nutrient utilization and energy expenditure. *Cell Metab* **17**, 491-506.

Mairbäurl H (2013) Red blood cells in sports: effects of exercise and training on oxygen supply by red blood cells. *Front Physiol* **4**, 332

Miller B, Kim SJ, Kumagai H, Mehta HH, Xiang W, Liu J, Yen K, Cohen P (2020). Peptides derived from small mitochondrial open reading frames: Genomic, biological, and therapeutic implications. *Exp Cell Res*. **393**, 112056

Mishmar D, Ruiz-Pesini E, Golik P, Macaulay V, Clark AG, Hosseini S, Brandon M, Easley K, Chen E, Brown MD, Sukernik RI, Olckers A, Wallace DC (2003) Natural selection shaped regional mtDNA variation in humans. *Proc Natl Acad Sci USA*. **100**, 171-6.

Mitchell P (1961) Coupling of Phosphorylation to Electron and Hydrogen Transfer by a Chemi-Osmotic type of Mechanism. *Nature* **191**, 144–148.

Nandy B, Gupta V, Sen S, Udaykumar N, Samant MA, Ali SZ, Prasad NG (2013) Evolution of mate-harm, longevity and behaviour in male fruit flies subjected to different levels of interlocus conflict. *BMC Evol Biol.* **13**, 212.

Nicholls DG, Locke RM (1984) Thermogenic mechanisms in brown fat. *Physiol Rev*. **64**, 1-64.

Nowack J, Giroud S, Arnold W, Ruf T (2017) Muscle Non-shivering Thermogenesis and Its Role in the Evolution of Endothermy. *Front Physiol.* **8**, 889.

Nystrand M, Cassidy EJ, Dowling DK (2018) The effects of a bacterial challenge on reproductive success of fruit flies evolved under low or high sexual selection. *Ecol Evol.* **8**, 9341-9352.

Olsson M, Madsen T, Shine R (1997) Is sperm really so cheap? Costs of reproduction in male adders, *Vipera berus*. *Proc. R. Soc. Lond. B.* **264**, 455-459.

Parker G (1979) Sexual selection and sexual conflict. In *Sexual Selection and Reproductive Competition in Insects*. eds. Blum, M. S. & Blum, N. A. (New York: Academic Press): 123-166.

Parker GA (2020) Conceptual developments in sperm competition: a very brief synopsis. *Phil Trans. R. Soc. B* **375**, 20200061.

Pizzari T, Parker GA (2009) Sperm competition and sperm phenotype. In *Sperm Biology: An Evolutionary Perspective*. (Academic Press), 215-245.

Rice WR, Holland B (2005) Experimentally enforced monogamy: inadvertent selection, inbreeding, or evidence for sexually antagonistic coevolution? *Evolution* **59**, 682–685

Rice WR, Stewart AD, Morrow EH, Linder JE, Orteiza N, Byrne PG (2006) Assessing sexual conflict in the Drosophila melanogaster laboratory model system. *Philos Trans R Soc Lond B Biol Sci.* **361**, 287-99.

Rowe L, Houle D (1996) The lek paradox and the capture of genetic variance by condition dependent traits. *Proc. R. Soc. Lond. B.* **263**, 1415-1421.

Shielzeth H, Dingemanse NJ, Nakagawa S, Westneat DF, Allegue H, Teplitsky C, Réale D, Dochtermann NA, Garamszegi LZ, Araya-Ajoy YG (2020) Robustness of linear mixed-effects models to violations of distributional assumptions. *Methods in Ecology and Evolution* **11**, 1141-1152

Simard CJ, Pelletier G, Boudreau LH, Hebert-Chatelain E, Pichaud N (2018) Measurement of Mitochondrial Oxygen Consumption in Permeabilized Fibers of *Drosophila* Using Minimal Amounts of Tissue. *J Vis Exp*. **7**, 57376.

Smith E, Morowitz H (2016) *The Origin and Nature of Life on Earth: The Emergence of the Fourth Geosphere*. Cambridge University Press.

Snook RR (2005) Sperm in competition: not playing by the numbers. *Trends Ecol Evol.* **20**, 46-53.

Snook RR, Brüstle L, Slate J (2009) A test and review of the role of effective population size on experimental sexual selection patterns. ***63***, 1923-33.

Speakman JR, Talbot DA, Selman C, Snart S, McLaren JS, Redman P, Krol E, Jackson DM, Johnson MS, Brand MD (2004) Uncoupled and surviving: individual mice with high metabolism have greater mitochondrial uncoupling and live longer. *Aging Cell.* **3**, 87-95.

Somjee U, Woods HA, Duell M, Miller CW (2018) The hidden cost of sexually selected traits: the metabolic expense of maintaining a sexually selected weapon. *Proc. R. Soc. B.* **285**, 20181685.

Storz JF, Bridgham JT, Kelly SA, Garland T Jr. (2015) Genetic approaches in comparative and evolutionary physiology. *Am J Physiol Regul Integr Comp Physiol.* **309**, 197-214.

Tarka M, Guenther A, Niemelä PT, Nakagawa S, Noble DWA (2018) Sex differences in life history, behavior, and physiology along a slow-fast continuum: a meta-analysis. *Behav Ecol Sociobiol.* **72**, 132.

Watson PJ, Arnqvist G, Stallmann RR (1998) Sexual conflict and the energetic costs of mating and mate choice in water striders. *Am Nat.* **151**, 46-58.

4.7 | FIGURES

Table 1. Results of a linear mixed effects model describing the effects of sexual selection strength (mating treatment), sex and their interaction on respiratory control ratio, PC1 scores (coupled respiration) and PC2 scores (uncoupled respiration). F statistics (calculated with Kenward-Rogers degrees of freedom) are reported along with p-values calculated from a Type III ANOVA. Respirometer chamber and a sex x replicate interaction were modelled as random effects. Estimated SD of random effects and residual values are included.

Fixed effects	Numerator df	Denominator df	F
	RCR		
Mating treatment	1	11.99	**6.35* (p = 0.027)**
Sex	1	11.97	**5.99* (p = 0.031)**
Mating treatment x Sex	1	11.94	0.29 (p = 0.599)
	PC1		
Mating treatment	1	11.94	0.56 (p = 0.467)
Sex	1	11.64	3.01 (p = 0.109)
Mating treatment x Sex	1	11.59	0.51 (p = 0.49)
	PC2		
Mating treatment	1	11.99	4.27 (p = 0.061)
Sex	1	11.97	4.3 (p = 0.06)
Mating treatment x Sex	1	11.95	0.09 (p = 0.764)

Random effects (SD)	Sex x Replicate	Respirometer chamber	Residual
RCR	0.11	0.055	0.12
PC1	0.24	0.89	2.06
PC2	0.574	0.13	0.91

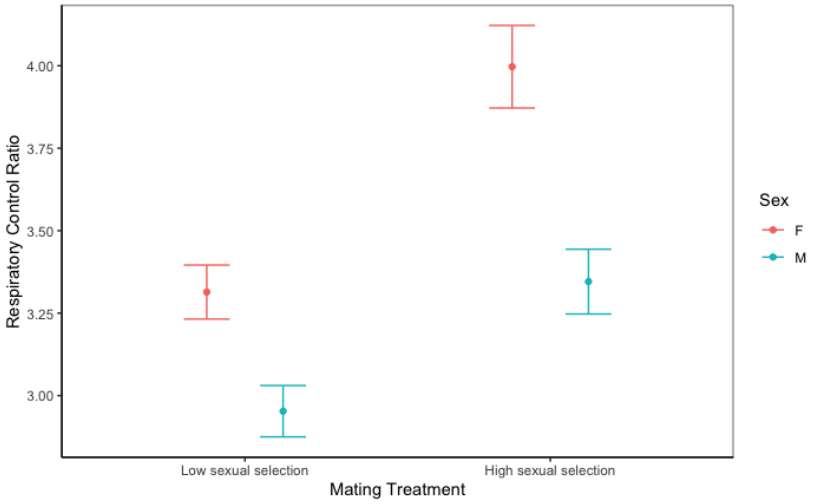

Figure 1. Respiratory control ratio in populations allowed to evolve under experimentally enforced high- and low- sexual selection mating treatments. Populations exposed to weaker sexual selection displayed significantly decreased respiratory control ratios, suggesting that they evolved lower respiratory efficiency. Females showed significantly higher RCR than males.

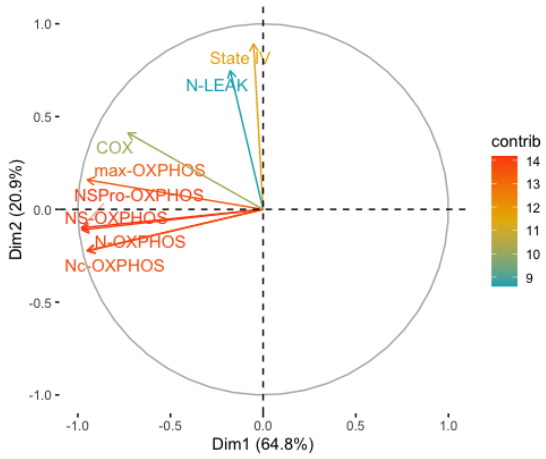

Figure 2. Principal component analysis (PCA) of respiratory parameters across eight experimentally evolved populations of *Drosophila melanogaster*. Principal component 1 (PC1) accounted for 64.8% of the variance in the respiratory parameters, with parameters representing coupled respiration (N-OXPHOS, NPro-OXPHOS, NProS-OXPHOS, max-OXPHOS) loading heavily onto this axis. The next principal component (PC2) accounted for 20.9% of the variance and was most strongly associated with parameters that represent uncoupled respiration (through either ADP insufficiency or ATP synthase inhibition - N-LEAK, State IV and COX).

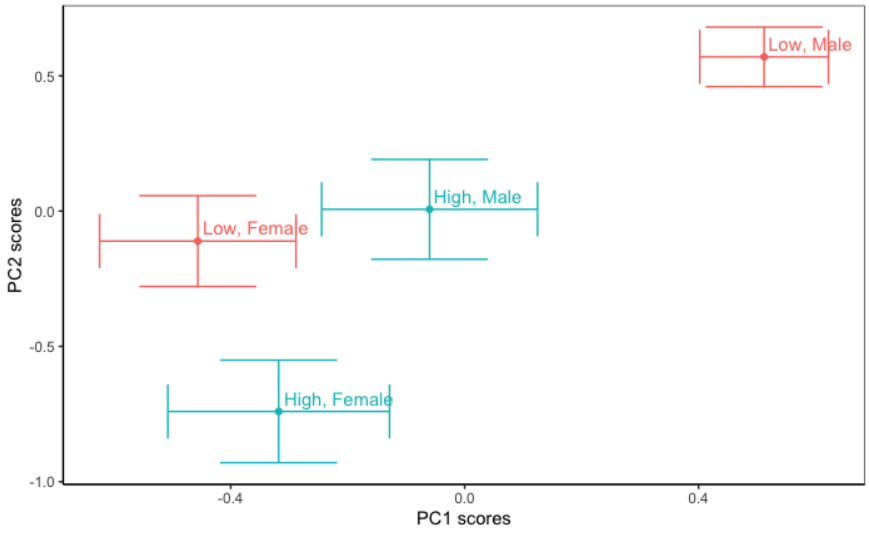

Figure 3. Effects of mating treatment and sex on scores in the first two major dimensions of variation in bioenergetic parameters. PC1 scores largely reflect oxygen consumption during coupled respiration, with higher scores indicating lower oxygen consumption. PC2 scores largely reflect oxygen consumption during uncoupled respiration, with higher scores representing higher oxygen consumption. Despite visual differences between groups of sex and mating treatment, there were no significant effects of mating treatment or sex on either of the principal components.

CHAPTER V | General Discussion

5.1 | DISCUSSION AND FUTURE DIRECTIONS

The birth of eukaryotes marked a distinct transition in the ecology of the electron transport system and the process of oxidative phosphorylation. Whereas genes controlling the structure and function of the ETS in prokaryotes are housed within a single genome, they are spread across two genomes in eukaryotes. Each of these genomes has different modes of inheritance, and may remain in only loose linkage disequilibrium (Eyre-Walker 2017), rendering maintenance of optimal OxPhos function in eukaryotes a complex control problem. In bilaterian metazoans, energetic demands are high, meaning that adequate control over the electron transport system and oxidative phosphorylation is particularly important. This thesis focused on OxPhos in *Drosophila melanogaster* and theoretical metapopulations of mitochondria, with a view to understanding how adequate control is maintained more broadly by bilaterian metazoans and under what conditions it may be

expected to fail. In chapter II, I took a theoretical approach to explore the evolution of mechanisms that help maintain well-functioning combinations of alleles across both mitochondrial and nuclear genomes. In chapter III, I explored how variation in OxPhos function is partitioned across the two genomes, and whether levels of genetic divergence common to wild populations are sufficient to induce mitonuclear interactions. In chapter IV, I addressed how bioenergetic function could be adaptively refined through increased pressures imposed by additional layers of selection that have evolved in bilaterian metazoans, such as sexual selection. In this chapter, I will provide an overview of the main findings from each research aim, discuss their significance relative to each other, and use these insights to discuss avenues for future research.

5.1.1 | Mechanisms that optimise mitonuclear match depend on ecological setting and magnitude of mitonuclear epistasis

My first aim was to gain a more nuanced understanding of the kind of processes that facilitate optimal mitochondrial function. Prior research into this question has focused on the uniparental inheritance (UPI) of mitochondrial genetic material; a process which is thought to have evolved due to the associated increase in speed of fixation of beneficial mtDNA alleles and elimination of deleterious mtDNA alleles, including 'selfish' mutants (Hoekstra 1990; Cosmides & Tooby 1991; Christie & Beekman 2017; Radzvilavicius 2021). While the adaptive value of UPI may apply to most eukaryotes, there are widespread and persistent exceptions that require explanation (Kvist *et al*. 2003; Welch *et al*. 2006; Pearl *et al*. 2009; Morgan *et al*. 2013; Radojicic *et al*. 2015; Mastrantonio *et al*. 2019a; Mastrantonio

et al. 2019b; Packert *et al*. 2019). In chapter II, I show that UPI may not be the optimal mtDNA inheritance strategy in hybridising populations where hybrids experience mitonuclear incompatibilities. I used a simulation-based, population genetic framework to show that a hypothetical 'mate-specific biparental inheritance' allele can out-compete a strict UPI allele and invade hybridising populations; particularly when mitonuclear incompatibilities are weaker and gene flow is subsequently higher. The model suggests that BPI offers an advantage by ensuring that a complete mitonuclear mismatch is exceedingly rare. In other words, BPI acts as a risk-avoidance strategy where maximum possible fitness is sacrificed to avoid minimum possible fitness. According to the model, this low-variance strategy can outcompete the high-variance UPI strategy when levels of gene flow are high enough to create a material risk of encountering the lowest-possible fitness genotype.

This work offers a possible explanation for the persistence of heteroplasmy in clinal regions where populations hybridise. While initial biparental inheritance of mitochondria may occur due to a failure of mitochondrial recognition and destruction systems (Ladoukakis & Zouros 2017, Sato & Sato 2017), the model predicts that this incidental BPI should not experience negative selection; that is, selection to re-instantiate uniparental inheritance. Thus, we may expect mate-specific BPI to persist for as long as populations remain in a state of hybridisation. The model also offers information about the kinds of hybridising systems where we may expect to find heteroplasmy due to BPI. One important prediction to come from the model is that BPI is most beneficial when selection coefficients against mitonuclear incompatibilities are low. In comparison to hybrids with severe mitonuclear incompatibilities, hybrid systems where incompatibilities are minor may be more likely to persist in a state of hybridisation since the participating populations are less likely to have

strong reproductive barriers in place. The type of mitonuclear incompatibilities that the model predicts to favour BPI may be maintained by spatial structuring of mitochondrial haplotypes. This has been shown to occur along clines, where environmental variables covary with haplotype frequencies (Camus *et al*. 2017; Lajbner *et al*. 2018; Rank *et al*. 2020). For example, populations of the Montane Leaf Beetle show haplotype segregation along altitudinal gradients, with admixture of mitochondrial and nuclear genotypes from extremes of the range resulting in mitonuclear incompatibilities that affect reproductive success (Rank *et al*. 2020). Heteroplasmy due to bi-parental inheritance of mitochondria has not yet been detected in this system.

A valuable test of the theory put forward in the present thesis would include assessing levels of heteroplasmy in clinal systems such as the Montane Leaf Beetle. However, it must be noted that the theory does not itself predict the *presence* of BPI in such populations, but the *persistence* of BPI. Thus, it would be interesting to know whether recognition mechanisms that enforce mtDNA destruction in UPI tend to fail before or after enough divergence has occurred for mitonuclear incompatibilities to be observed. Our model would predict that mechanisms enforcing UPI should evolve more rapidly than drift, resulting in failure and subsequent biparental inheritance of mitochondria upon secondary contact. This would, however, require sampling very many hybrid populations. Perhaps a more informative study would assess rates of heteroplasmy attributable to BPI in populations that are known to be stable, long-standing hybrid populations. The model would predict that, in such populations, reproductive barriers would evolve before mtDNA recognition and destruction systems evolve.

Perhaps most importantly, the model suggests that BPI will be favoured over UPI in a system of hybridising populations where mitonuclear interactions predominate over mitochondrial main effects. Though additive variation in mtDNA was not explicitly modelled in chapter II, theory holds that if a particular mitochondrial haplotype is beneficial regardless of nuclear background, uniparental inheritance will facilitate more rapid fixation of the allele (Christie & Beekman 2017). In chapter III, I showed that when closely related mitochondrial haplotypes are experimentally introgressed alongside various nuclear backgrounds, additive effects on coupled respiration are larger than epistatic interactions. Thus, the two studies in combination afford the inference that, at least for bilaterian metazoans, UPI is likely more common than BPI at least partly because additive effects of mtDNA variation predominate over epistatic effects in many populations. Although the model predicts that weak selection against mitonuclear incompatibilities facilitate spread of a hypothetical biparental inheritance allele, mitonuclear interactions are still the major determinant of fitness (over and above additive mitochondrial variation). It may be that mitonuclear epistasis within populations is never sufficiently large in magnitude *relative to additive* mitochondrial variation to favour BPI. Further work incorporating both mitonuclear epistasis and additive mitochondrial variation into theoretical models, and understanding the consequences of varying their *relative* contributions may shed light on this hypothesis.

5.1.2 | Mitonuclear epistasis does not outweigh mtDNA additive effects when divergence between haplotypes is comparable to wild, interbreeding populations

In chapter III, I addressed the relative impact of nuclear genetic variation, mitochondrial genetic variation, and their interaction on OxPhos function. My aim was to gain a deeper understanding of how control over OxPhos is partitioned across the two genomes in

bilaterian metazoans, using *D. melanogaster* as a model species. I leveraged a full-factorial panel of strains that mixed and matched nuclear and mitochondrial genotypes derived from three separate allopatric populations, and took high resolution measurements of oxygen consumption in various states of ETS activation from adult flies. Using this approach, I was able to identify two primary dimensions of variation in OxPhos function that roughly corresponded to coupled (ATP producing) and uncoupled (non ATP-producing) respiration. In addition to the two primary dimensions of variation, I assessed the respiratory control ratio; a metric of respiratory efficiency. I showed that both nuclear and mitochondrial genetic variation affects coupled respiration in an additive fashion, with mitochondrial genetic variation exerting a larger effect. Nuclear genetic variation strongly affected both uncoupled respiration and RCR ratio; both in an additive fashion and in concert with age and sex. Mitonuclear epistasis contributed significantly toward measures of uncoupled respiration, though the magnitude of this effect was considerably smaller than the effect of nuclear genetic variation alone.

Given the small number of genes housed in the mitochondria compared to the number of mitochondrial-related genes housed in the nucleus (Wolstenholme 1992; Lotz *et al*. 2014), the mtDNA haplotype exerts a large relative effect on coupled respiration. Such a finding offers valuable insights into one of the deeper questions regarding the dual genomic control over OxPhos in bilaterian metazoans: why have mitochondria retained genes at all? It may seem, at face value, that housing the complete set of requisite genes in the nucleus may minimise errors in the ETS by allowing natural selection to work more efficiently on a group of genes that can exist in linkage disequilibrium (Kirkpatrick 2010). Various hypotheses have been presented as solutions to the problem. Two opposing (though not exhaustive)

hypotheses hold that (i) genes have been (and continue to be) lost randomly and uniformly (Rispe & Moran 2000; Berg & Kurland 2000) and (ii) that genes central to energy production (that is, genes encoding proteins of the ETS with high interaction energy) are preferentially retained in the mitochondria (Allen 2015; Johnston & Williams 2016). In chapter III, I was able to provide an empirical demonstration that genes considered by theory to encode proteins with high 'interaction energies' (that is, those remaining in the mitochondrial genome according to the latter hypothesis) do in fact shape coupled respiration, and further, they do so with an effect size equivalent to the nuclear genome. While this evidence does not disprove theories of random and uniform loss of mitochondrial genes, it does stand as a demonstration of a key necessary condition of the hypothesis that 'energetically central' genes have been preferentially retained.

Broadly, the work presented in chapter III positions mitochondrial and nuclear genetic variation above the mitonuclear interaction in terms of relative importance regarding control over the ETS and OxPhos function. Such a finding lies in contrast to a strong compensatory co-evolution model, which would predict even mildly divergence between populations to result in dominant mitonuclear incompatibilities (Gershoni *et al*. 2009; Burton & Barreto 2012). This model assumes that most mitochondrial substitutions are detrimental, requiring nuclear co-evolution to 'rescue' function. My work challenges this assumption, instead suggesting that much of the mutation load of mtDNA can be screened out by purifying selection before the need arises for nuclear compensation. Indeed, the many levels of selection acting on mtDNA and repeated bottlenecking characteristic of oogenesis and uniparental inheritance of mitochondria allow for far more efficient purifying selection than was previously thought (Cree *et al*. 2008; Fan *et al*. 2013; Hill *et al*. 2019;

Stewart & Larsson 2014; Camus & Dhawanjewar 2023). If detrimental mutations are purified before they can exert selective pressures on the nuclear genome, the phenotypic variation attributable to mtDNA in chapter III may be best explained as adaptive variation. Indeed, across all metazoans, up to 25% of mutations in the mitochondrial genome are thought to be adaptive (James *et al*. 2016) and experimental evidence demonstrates that mtDNA variants contribute to climate adaptation (Mishmar *et al.* 2003; Ruiz-Pesini *et al*. 2004; Camus *et al*. 2017; Lajbner *et al*. 2018). Adaptive mutation in mtDNA may not place the kind of extreme selective pressure on the nuclear genome that would be expected to produce a compensatory response. In keeping with our findings, if nuclear genomes are not co-evolving strongly in response to mtDNA variation, we would expect to observe mtDNA variation acting in an additive fashion. Thus, my work suggests that adaptive mtDNA mutations may produce *positive* phenotypic consequences that outweigh the *negative* phenotypic consequences, in such a way that nuclear compensatory evolution takes on lower selective importance.

Although small in effect size, mitonuclear interactions were responsible for contributing to variation in uncoupled respiration. The pattern of mitonuclear interaction seems to suggest that it is driven by relatively rare functional epistasis. In other words, only one of the nine strains showed unusual deviations in uncoupled respiration. This could, however, equally be interpreted as incompatibilities in *two* of the three mitonuclear pairings with the Zimbabwe nuclear background, suggesting a tight co-adaptation between the ZIM nuclear and mitochondrial genomes. Such an interpretation would instead imply that functional epistasis may be more common; even ubiquitous, if only minor in its effect. Regardless of interpretation, more work needs to go into understanding what factors increase the

likelihood of an incompatibility. Currently, in crosses of populations with sequence divergence representative of wild, interbreeding populations, the pattern of functional epistasis is unclear and does not correlate with genetic distance (Camus *et al*. 2020). We suggest that future work should focus on finding similarities across strains exhibiting functional epistasis. It will be important to establish which (if any) genes are commonly involved, whether incompatibilities are concentrated in proteins or tRNAs, and finally whether changes to these polypeptides have similar morphological or functional characteristics.

5.1.3 | Sexual selection acts on mitochondrial bioenergetics

My final aim was to ascertain whether OxPhos function is influenced by sexual selection. Despite theory predicting that capacity to produce an abundance of usable energy should favour performance in sexually selected traits, few studies have explicitly linked metabolic rate or bioenergetic parameters with reproductive success. Recent work has shown that sexual selection shapes the evolution of whole-animal metabolic rate (Garlovsky 2022) and further work has linked attractiveness in birds to variation in the respiratory control ratio (Hill *et al*. 2019). This work suggests that intense sexual selection should lead to changes in respiratory control ratio, and that this may (or may not) be accompanied by increased maximum electron flux through the ETS. In chapter IV, I show that differences in the strength of sexual selection consistently leads to the evolution of directional changes in the respiratory control ratio, without associated changes in maximum ETS flux. This finding suggests that high sexual selection is associated with increased bioenergetic efficiency.

In demonstrating that bioenergetic performance is shaped by sexual selection, my work ultimately opens several further lines of enquiry regarding how, precisely, this selection takes place. Firstly, it is unclear whether the responses of RCR to selection were mediated primarily by differences male-male competition across the treatments, female choice, any of the various forms of post-copulatory selection, or a combination of all of them. Resolving this uncertainty will require further studies linking RCR with each form of selection (or even each trait) in isolation, such as fighting capacity, attractiveness, and sperm number/function. These studies may take the form of assessing standing variation in outbred populations, mate choice experiments on the experimental evolution populations discussed in the present work, or experimental manipulations involving uncoupling agents.

Secondly, it is unclear whether the increased RCR observed in my study is beneficial to both males and females or subject to sexual conflict, where one sex benefits at a cost to the other. My work in chapter III shows clearly that there are strong sex differences in RCR. Given the high likelihood that genes controlling RCR are nuclear autosomal (Chapter III), and thus shared by the two sexes, it is possible that the changes observed in chapter IV are driven by stronger sexual selection in one of the sexes, at the expense of fitness of the other (Trivers 1972; Parker 1979; Bonduriansky & Chenoweth 2009). Experimental evolution systems can be manipulated such that only one sex is the focus of sexual selection (Bennet & Lenski 1999; Holland & Rice 1999). In these cases, if trait changes in the sex not under sexual selection pressures are associated with lower fitness, we may infer that the trait has evolved under intralocus sexual conflict. Such studies present an opportunity to clarify the nature of the intersexual dynamics acting to shape RCR trait values. However, the fact that prior work on the experimentally evolved populations used in Chapter IV has demonstrated

increased measures of fitness in each of the sexes (Innocenti *et al*. 2014; Nystrand *et al*. 2018) suggests that the increased RCR associated with strong sexual selection may not be detrimental for either of the sexes.

A second question raised from chapter IV is whether selection is occurring on the nuclear or mitochondrial genome. Theory would predict that the nuclear genome is the most likely candidate since males do not pass on their mtDNA to their offspring. If changes in RCR were enacted through the mitochondrial genome, then the only plausible form of sexual selection through which they could occur is male choice of 'high quality' females. Though male-choice is known to be a considerable selective force in *D. melanogaster* (Byrne & Rice 2006), it is unlikely stronger than both male-male competition and female-choice in concert, both of which could exert selection on the mitochondrial genome, but could not bring about an evolutionary response (Frank & Hurst 1996; Innocenti *et al*. 2011). It is important to note, however, that there was likely little or no mtDNA variation within the populations used in the experiment. This may have limited the opportunity for any selection at all to act on mtDNA. As noted earlier, sexual selection on mtDNA could occur through male-choice, and explicitly incorporating variation in mtDNA in order to understand whether mtDNA is subject to sexual selection remains an avenue for future research.

Findings from chapter III and chapter IV, together, support the notion that the nuclear genome is responsible for the changes in RCR between populations of different mating treatments. This is because, in chapter III, RCR is shown to be under nuclear control. Chapter III also reveals that RCR is subject to an interaction between nuclear genotype and age, suggesting that performance under sexual selection may in fact change with age

differently across varying nuclear backgrounds. In other words, sex- and age- specific variation in RCR, together with an evolutionary response in RCR to sexual selection, supports a model whereby allocation to performance in sexually selected traits is subject to pleiotropic trade-offs: higher RCR in early life may facilitate greater allocation to sexually selected traits, but may result in differential rates of change in the trait. Indeed, prior work has shown links between lifespan and the strength of sexual selection, suggesting that strong sexual selection favours a 'live fast, die-young' phenotype (Nandy *et al*. 2013; Tarka *et al*. 2018; Arnqvist *et al*. 2022), where organisms allocate resources to sexually selected traits at the cost of longevity (Hunt *et al*. 2004).

My work suggests that optimal RCR rates depend on ecological setting: it is not simply a case of 'higher is always better'. Given this insight, it is unlikely that there is universal directional selection for higher ATP yields per oxygen molecule (and fuel). Going forward, a goal of the field should be to understand which situations favour tighter coupling and which favour lower coupling. Furthermore, it may be that pressures favouring one direction or the other are not consistent over entire lifespans of organisms. Given that higher RCR states are associated with higher ROS production (Skulachev 1996; Brand 2000), and ROS levels are under tight regulatory control (Ray *et al*. 2012), RCR levels, too, are likely under tight regulatory control. In fact, mitochondrial coupling, a key determinant of RCR, is known to be plastic over short timespans (Brand 2005; Demine *et al*. 2019). While demonstrating in the present thesis that higher sexual selection increases RCR *on average*, an avenue for future work includes understanding what factors contribute to the variance around this average. Furthermore, it may be insightful to understand whether absolute rates, or rather speed

and efficacy of plastic responses to shifting ecological and cellular conditions is the primary determinant of fitness in organisms.

5.2 | CONCLUSION

The work in this thesis has explored just some of the ways that evolution has ensured organisms extract the energy they need to survive and reproduce. The oxidative phosphorylation system in bilaterian metazoans is subject to particular ecological dynamics relative to other eukaryotes and all prokaryotes. I have showed that, far from transferring all control to the nuclear genome, functionally important genes that contribute substantially to variation in OxPhos remain in the mitochondrial genome. When haplotypes are placed alongside putatively mismatched nuclear genomes derived from recently diverged allopatric populations, main effects of mitochondrial haplotype predominate, along with nuclear genotype, over the interaction between the two genomes. This stands in contrast to models of distantly related hybridising populations where any mitochondrial additive variation may not be expressed alongside distantly related nuclear genotypes, and thus the interaction may be relatively more impactful. Depending on whether epistasis or additive effects dominate in any system, we may see different mechanisms evolving in place to ensure optimal mitochondrial function. In other words, the way that mitochondria are inherited offer one way that organisms can respond adaptively to pressures for adequate mitochondrial function. My work shows that in hybridising populations, where epistasis dominates over additive mitochondrial effects, we may predict that biparental inheritance of mitochondria offers an adaptive advantage over uniparental inheritance (UPI). Finally, my work demonstrates that a further layer of selection supporting and shaping mitochondrial function in bilaterian metazoans is sexual selection. Genes controlling the efficiency with

which mitochondria produce energy are subject to evolution under variation in the strength of sexual selection, with stronger sexual selection supporting evolution of higher efficiency, albeit at the potential cost of shortened lifespan. Together, the dual genomic control over the ETS, the way that mtDNA inheritance patterns may provide context-specific benefits, and the way that bilaterian metazoans may evolve to leverage signals of mitochondrial function to inform mating decisions, all provide unique perspectives into how far the process of passing of electrons down a chain of proteins has come since its humble prokaryotic beginnings.

5.3 | REFERENCES

Allen JF (2015) Why chloroplasts and mitochondria retain their own genomes and genetic systems: Colocation for redox regulation of gene expression. *Proc Natl Acad Sci USA* **112**, 10231-8.

Arnqvist G, Rönn J, Watson C, Goenaga J, Immonen E (2022) Concerted evolution of metabolic rate, economics of mating, ecology, and pace of life across seed beetles. *Proc Natl Acad Sci USA.* **119**, e2205564119.

Bennett AF, Lenski RE (1999) Experimental evolution and its role in evolutionary physiology. *Am. Zool.* **39**, 346–362.

Berg OG, Kurland CG (2000) Why mitochondrial genes are most often found in nuclei. *Mol Biol Evol.* **17**, 951-61.

Bonduriansky R, Chenoweth SF (2009) Intralocus sexual conflict. *Trends Ecol Evol.* **24**, 280-8.

Brand MD (2000) Uncoupling to survive? The role of mitochondrial inefficiency in ageing. *Exp Gerontol.* **35**, 811-20.

Brand MD (2005) The efficiency and plasticity of mitochondrial energy transduction. *Biochem Soc Trans*. **33**, 897-904.

Burton RS, Barreto FS (2012) A disproportionate role for mtDNA in Dobzhansky-Muller incompatibilities? *Mol Ecol*. **21**, 4942-57.

Byrne PG, Rice WR (2006) Evidence for adaptive male mate choice in the fruit fly Drosophila melanogaster. *Proc Biol Sci.* **273**, 917-22.

Camus MF, Wolff JN, Sgrò CM, Dowling DK (2017) Experimental Support That Natural Selection Has Shaped the Latitudinal Distribution of Mitochondrial Haplotypes in Australian Drosophila melanogaster. *Mol Biol Evol.* **34**, 2600-2612.

Camus MF, Dhawanjewar AS (2023) Multilevel selection on mitochondrial genomes. *Curr Opin Genet Dev*. **80**, 102050.

Camus MF, O'Leary M, Reuter M, Lane N (2020) Impact of mitonuclear interactions on life-history responses to diet. *Philos Trans R Soc Lond B Biol Sci*. **375**, 20190416.

Christie JR, Beekman M (2017) Uniparental inheritance promotes adaptive evolution in cytoplasmic genomes. *Mol. Biol. Evol.* **34**, 677-691.

Cosmides LM, Tooby J (1981) Cytoplasmic inheritance and intragenomic conflict. *J Theor Biol.* **89**, 83-129.

Cree LM, Samuels DC, de Sousa Lopes SC, Rajasimha HK, Wonnapinij P, Mann JR, Dahl HH, Chinnery PF (2008) A reduction of mitochondrial DNA molecules during embryogenesis explains the rapid segregation of genotypes. *Nat Genet.* **40**, 249-54.

Demine S, Renard P, Arnould T (2019) Mitochondrial Uncoupling: A Key Controller of Biological Processes in Physiology and Diseases. *Cells* **8**, 795.

Eyre-Walker A (2017) Mitochondrial Replacement Therapy: Are mito-nuclear interactions likely to be a problem? *Genetics* **205**, 1365-1372

Fan W, Waymire KG, Narula N, Li P, Rocher C, Coskun PE, Vannan MA, Narula J, Macgregor GR, Wallace DC (2008) A mouse model of mitochondrial disease reveals germline selection against severe mtDNA mutations. *Science* **319**, 958-62.

Frank S, Hurst L (1996) Mitochondria and male disease. *Nature* **383**, 224

Garlovsky MD, Holman L, Brooks AL, Novicic ZK, Snook RR (2022) Experimental sexual selection affects the evolution of physiological and life-history traits. *J Evol Biol* **35**, 742-751.

Gershoni M, Templeton AR, Mishmar D (2009) Mitochondrial bioenergetics as a major motive force of speciation. *Bioessays* **31**, 642-50.

Hill GE, Hood WR, Ge Z, Grinter R, Greening C, Johnson JD, Park NR, Taylor HA, Andreasen VA, Powers MJ, Justyn NM, Parry HA, Kavazis AN, Zhang Y (2019) Plumage redness signals mitochondrial function in the house finch. *Proc Biol Sci.* **286**, 20191354.

Hoekstra RF (1990) Evolution of uniparental inheritance of cytoplasmic DNA. In *Organisational constraints on the dynamics of evolution* (eds Maynard-Smith J, Vida J). Manchester, UK: Manchester University Press.

Holland B, and Rice WR (1999) Experimental removal of sexual selection reverses intersexual antagonistic coevolution and removes a reproductive load. *Proc. Natl. Acad. Sci. USA* **96**, 5083–5088.

Hunt J, Brooks R, Jennions MD, Smith MJ, Bentsen CL, Bussière LF (2004) High-quality male field crickets invest heavily in sexual display but die young. *Nature* **432**, 1024-7.

Innocenti P, Morrow EH, Dowling DK (2011) Experimental evidence supports a sex-specific selective sieve in mitochondrial genome evolution. *Science* **332**, 845-8.

Innocenti P, Flis I, Morrow EH (2014) Female responses to experimental removal of sexual selection components in Drosophila melanogaster. *BMC Evol Biol*. **14**, 239.

Johnston IG, Williams BP (2016) Evolutionary Inference across Eukaryotes Identifies Specific Pressures Favoring Mitochondrial Gene Retention. *Cell Syst.* **2**, 101-11.

Kirkpatrick M (2010) How and why chromosome inversions evolve. *PLoS Biol.* **8**, e1000501.

Kvist L, Martens J, Nazarenko AA, Markku O (2003) Paternal leakage of mitochondrial DNA in the Great Tit (*Parus major*). *Molecular Biology and Evolution* **20**, 243-247.

Ladoukakis ED, Zouros E (2017) Evolution and inheritance of animal mitochondrial DNA: rules and exeptions. *Journal of Biological Research-Thessaloniki* **24**: 2

Lajbner Z, Pnini R, Camus MF, Miller J, Dowling DK (2018) Experimental evidence that thermal selection shapes mitochondrial genome evolution. *Sci Rep*. **8**, 9500.

Lotz C, Lin AJ, Black CM, Zhang J, Lau E, Deng N, Wang Y, Zong NC, Choi JH, Xu T, Liem DA, Korge P, Weiss JN, Hermjakob H, Yates JR 3rd, Apweiler R, Ping P (2014) Characterization, design, and function of the mitochondrial proteome: from organs to organisms. *J Proteome Res.* **13**, 433-46.

Mastrantonio V, Latrofa MS, Porretta D, Lia RP, Parisi A, Latta R, Dantas-Torres F, Otranto D, Urbanelli S (2019a) Paternal leakage and mtDNA heteroplasmy in *Rhipicephalus* spp. Ticks. *Scientific Reports* **9**, 1460.

Mastrantonio V, Urbanelli S, Porretta D (2019b) Ancient hybridisation and mtDNA introgression behind current paternal leakage and heteroplasmy in hybrid zones. *Scientific Reports* **9**, 19177.

Mishmar D, Ruiz-Pesini E, Golik P, Macaulay V, Clark AG, Hosseini S, Brandon M, Easley K, Chen E, Brown MD, Sukernik RI, Olckers A, Wallace DC (2003) Natural selection shaped regional mtDNA variation in humans. *Proc Natl Acad Sci USA*. **100**, 171-6.

Morgan JAT, Macbeth M, Broderick D, Whatmore P, Street R, Welch DJ, Ovenden JR (2013) Hybridisation, paternal leakage and mitochondrial DNA linearisation in three anomalous fish (Scombridae). *Mitochondrion* **13**, 852-861.

Nandy B, Gupta V, Sen S, Udaykumar N, Samant MA, Ali SZ, Prasad NG (2013) Evolution of mate-harm, longevity and behaviour in male fruit flies subjected to different levels of interlocus conflict. *BMC Evol Biol.* **13**, 212.

Nystrand M, Cassidy EJ, Dowling DK (2018) The effects of a bacterial challenge on reproductive success of fruit flies evolved under low or high sexual selection. *Ecol Evol.* **8**, 9341-9352.

Packert M, Giacalone G, Valvo ML, Kehlmaier C (2019) Mitochondrial heteroplasmy in an avian hybrid form (*Passer italia*: Aves, Passeriformes). *Mitochondrial DNA Part B* **4**, 3809-3812.

Parker G (1979) Sexual selection and sexual conflict. In *Sexual Selection and Reproductive Competition in Insects*. eds. Blum, M. S. & Blum, N. A. (New York: Academic Press): 123-166.

Pearl SA, Welch ME, McCauley DE (2009) Mitochondrial heteroplasmy and paternal leakage in natural populations of Silene vulgaris, a gynodioecious plant. *Molecular Biology and Evolution* **26**, 537-545.

Radojicic JM, Krizmanic I, Kasapidis P, Zouros E (2015) Extensive mitochondrial heteroplasmy in hybrid water frog (*Pelophylax* spp.) populations from Southeast Europe. *Ecology and Evolution* **5**, 4529–4541.

Radzvilavicius A (2021) Beyond the "selfish mitochondrion" theory of uniparental inheritance: A unified theory based on mutational variance redistribution. *Bioessays* **43**, e2100009.

Rank NE, Mardulyn P, Heidl SJ, Roberts KT, Zavala NA, Smiley JT, Dahlhoff EP (2020) Mitonuclear mismatch alters performance and reproductive success in naturally-introgressed populations of a montane leaf beetle. *Evolution* **74**, 1724-1740.

Ray PD, Huang BW, Tsuji Y (2012) Reactive oxygen species (ROS) homeostasis and redox regulation in cellular signaling. *Cell Signal.* **24**, 981-90.

Rispe C, Moran NA (2000) Accumulation of Deleterious Mutations in Endosymbionts: Muller's Ratchet with Two Levels of Selection. *Am Nat.* **156**, 425-441.

Ruiz-Pesini E, Mishmar D, Brandon M, Procaccio V, Wallace DC (2004) Effects of purifying and adaptive selection on regional variation in human mtDNA. Science **303**, 223-226.

Sato K, Sato M (2017) Multiple ways to prevent transmission of paternal mitochondrial DNA for maternal inheritance in animals. *Journal of Biochemistry* **162**, 247-253.

Skulachev VP (1996) Role of uncoupled and non-coupled oxidations in maintenance of safely low levels of oxygen and its one-electron reductants. *Q Rev Biophys*. **29**, 169-202

Stewart JB, Larsson NG (2014) Keeping mtDNA in shape between generations. *PLoS Genet*. **10**, e1004670.

Tarka M, Guenther A, Niemelä PT, Nakagawa S, Noble DWA (2018) Sex differences in life history, behavior, and physiology along a slow-fast continuum: a meta-analysis. *Behav Ecol Sociobiol.* **72**, 132.

Trivers RL (1972) Parental investment and sexual selection. In B. Campbell (Ed.), *Sexual selection and the descent of man* (pp. 136-179)

Welch ME, Darnell MZ, McCauley DE (2006) Variable Populations within variable populations: quantifying mitochondrial heteroplasmy in natural populations of the gynodioecious plant *Silene vulgaris. Genetics* **174**, 829–837.

Wolstenholme DR (1992) Animal mitochondrial DNA: structure and evolution. Int Rev Cytol. **141**, 173-216.