PROJECT TITLE: More than a powerhouse: unravelling the effects of mitochondrial genetic variation on physiology and behaviour

AIMS AND BACKGROUND

For many decades, it was assumed that sequence variation in the genome of the mitochondria (mitochondrial DNA, or mtDNA) did not alter phenotypic function, and was therefore neutral to natural selection (Galtier *et al.* 2009). Recent studies have challenged this assumption, showing that mitochondrial genetic variation can have significant effects on such major traits as fertility and lifespan in species as diverse as humans (Montiel-Sosa *et al.* 2006) and fruit flies (Camus *et al.* 2015). Currently, the underlying mechanisms that link mitochondrial genotype to phenotype remain elusive and in need of direct experimental attention.

One of the most surprising findings of recent experiments has been the discovery that mtDNA variation can affect male and female phenotype in different ways, even to the extent that heritable sequence variants (haplotypes) might enhance phenotypic function in females but decrease male function. (Camus & Dowling 2017). The "Mother's Curse" Hypothesis predicts that heritable, male-harming mutations will accumulate within the mtDNA sequence, because maternal inheritance of the mitochondrial genome should render natural selection ineffective at weeding out mutations of male-biased effect (Frank & Hurst 1996; Gemmell *et al.* 2004). While this hypothesis presents a sound *theoretical explanation* for the sexually antagonistic effects that have been attributed to mtDNA, the *mechanisms* by which mtDNA sequence can exert these opposing effects on males and females remain elusive.

The overarching aim of this proposal is to unravel the proximate mechanisms and ultimate consequences of mitochondrial genetic variation across three broad levels: molecular, physiological, and behavioural. I will test for population- and sex-specific differences across these three arenas.

Understanding how variation in mtDNA affects both mitochondrial function and organismal performance is timely and important because a growing body of research implicates mitochondria as critical not only to energy production, but also to a variety of other processes as disparate and fundamental as immune function and neural development (Koch *et al.* 2017; Koch & Hill 2018). However, the specific role of the mitochondrion's own genome in contributing to overall mitochondrial functionality and the performance of related processes is uncertain. Resolving how variation in the mitochondrial genome contributes to such major processes will have a tangible impact on how biomedical and ecological researchers alike study the genetic underpinnings of physiological variation, with clear applications to understanding differences among individuals and between the sexes.

I will accomplish the overarching goal of this project using genetic strains of the fruit fly (*Drosophila melanogaster*) with unprecedented power to map phenotypic variation to the level of population-specific mitochondrial DNA variation (different mitochondrial "haplotypes"). I will use this system to address three specific research objectives (Fig. 1):

Objective 1: Determine how mtDNA haplotype affects the MOLECULAR phenotype through changes to mitochondrial efficiency and oxidative stress.

Objective 2: Evaluate how mtDNA haplotype alters individual performance in specific PHYSIOLOGICAL processes that have been linked to mitochondrial function.

Objective 3: Test how mtDNA haplotype affects BEHAVIOURAL performance and reproductive success.

Importantly, I will test all three of these objectives across both sexes and with mtDNA haplotypes expressed alongside three different nuclear genomes. This will allow me to perform definitive tests of the Mother's Curse Hypothesis as well as how interactions between nuclear and mitochondrial DNA affect phenotypic variation.

My DECRA project integrates my past research into the physiological effects of variation in mitochondrial performance (Koch *et al.* 2017; Koch & Hill 2018; published under my maiden name, Koch) with current research that has established surprising patterns of functional, sex-specific mtDNA variation (e.g. Camus & Dowling 2017). These exciting new experiments will impact the understanding of processes as wide-ranging as sexual conflict and cellular evolution.

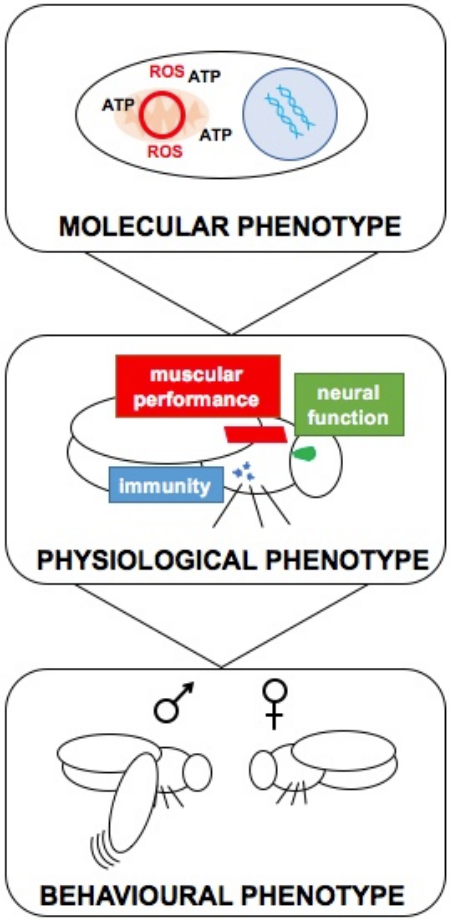


Figure 1. Illustration of the multiple levels of analysis in this proposal.

PROPOSED PROJECT QUALITY AND INNOVATION

Addressing conceptual problems with innovative solutions

Functional mitochondrial genetic variation: In animals, mitochondrial genes primarily code for proteins involved in the electron transport chain—the main source of energy-storing ATP within most animal cells (Hill 2015). Variation in mtDNA sequence that affects the structure of these proteins has major consequences for cellular performance, altering the rate of production of mitochondrial reactive oxygen species (both a signalling molecule and a source of harmful oxidative damage to cellular machinery; Sena & Chandel 2012). My proposed research will close a major gap in the current literature by exploring whether variation in mtDNA sequence alters phenotype through changes to oxidative stress. This proximate mechanism (oxidative stress) is a promising explanation for how mtDNA sequence differences can have ultimate effects on traits like lifespan or reproductive success (Dowling & Simmons 2009).

Importantly, genes coded in the nuclear genome are also involved in mitochondrial performance, and the epistatic interactions between these two genomes can alter phenotype in addition to direct effects of mtDNA variation alone ("mito-nuclear interactions"; Wolff *et al.* 2014). However, few studies have tested for such epistatic interactions, and existing tests of functional mtDNA variation have been restricted to testing either many different mtDNA haplotypes expressed alongside one standardized nuclear genome (called the "nuclear background" to the mtDNA), or few mtDNA haplotypes expressed alongside few nuclear backgrounds. Such methods sacrifice power to detect the effects of mito-nuclear interactions or mtDNA variation. Here, I develop a new system in which I replicate extensive mtDNA haplotype variation across three different nuclear backgrounds, a key innovation to allow unprecedented statistical power to test the effects of mtDNA variation as well as mito-nuclear interactions. Each of my experiments will therefore not only unravel patterns in phenotype due to mtDNA variation, but also test whether those patterns are consistent across nuclear backgrounds.

Sex-specific (Mother's Curse) effects: Although the mitochondrial genome is best known for its role in energy-producing cellular metabolism, recent studies suggest that genetic variants in mtDNA can have much more complex interactions with phenotype than changes to metabolism alone. The discovery of sex-specific effects of mtDNA variation that are consistent with Mother's Curse Hypothesis suggests that our understanding of mitochondrial pathways is incomplete and in need of urgent study, as we cannot yet explain how the mtDNA genome exerts sex-specific effects on important fitness traits like fertility.

An important component of the Mother's Curse Hypothesis is that the more sexually dimorphic a trait is, the more likely it will incur Mother's Curse effects. This is because traits that feature male-specific expression are rich ground for genetic mutations that affect only males, and therefore will not be eliminated through purifying selection on female mtDNA (Innocenti *et al.* 2011). The Mother's Curse Hypothesis therefore specifically predicts that sexually dimorphic, male-specific processes—like sperm production in the testes, or male-specific courtship behaviours—will be most likely to accumulate male-harming mutations. I can detect the presence of Mother's Curse effects by finding greater phenotypic variation among haplotypes in males than in females, which suggests the accumulation of varying degrees of male-biased mutations across haplotypes.

Mitochondrial function drives physiological function: In two recent synthesis papers, I review extensive literatures from biomedicine and neurobiology to argue that the performance of an individual's mitochondria drives variation in complex sensorimotor and cognitive processes (Koch & Hill 2018) and innate and adaptive immune system function (Koch *et al.* 2017). However, the implications of these links between mitochondria and fundamental physiological pathways have not yet been explored within an ecological or evolutionary context, and there is rich potential for mitochondrial genetics to affect mitochondria-reliant systems, potentially in a tissue- or sex-specific manner. Indeed, while I have previously posited that high quality mitochondrial performance may drive high quality physiological performance across a suite of related processes within an individual (positive pleiotropy), it is now clear mtDNA variants that are good for one process can be detrimental to another (antagonistic pleiotropy; Camus & Dowling 2017). In my proposed DECRA research, I will perform the first clear tests of how variation in mtDNA affects health-related traits—immune system function, locomotor performance, and cognitive ability—allowing me to test for positive or antagonistic pleiotropy.

Drosophila *mate choice:* The courtship display of male *D. melanogaster* integrates performance across the processes described above—specifically, motor performance (Greenspan & Ferveur 2000), tactical decision-making (Dukas 2005), and even immune system investment (McKean & Nunney 2001). Indeed, the neural and sensorimotor underpinnings of *D. melanogaster* courtship behaviours are characterized to an unprecedented extent, and have been found to rely on the performance of sex-specific neural pathways (Dickson 2008). These types of complex and intricate behaviours reliant on the careful integration of sensorimotor and neural processes are exactly those I have predicted to be highly dependent on mitochondrial function (Koch & Hill 2018). And, the sexual dimorphism of the pathways involved in generating the male courtship displays makes them a likely target of Mother's Curse mutations.

As such, my proposed research will test whether male *D. melanogaster* courtship display performance and mating success depends on mtDNA haplotype and the interactions of that mtDNA with the nuclear genome. Integrated together with the other components of this DECRA proposal, this will be the first experiment to directly test for links between mitochondrial performance (Obj. 1) to internal physiology (Obj. 2) and finally to sexually selected display quality (Obj. 3). Determining whether mtDNA genetic variation causes phenotypic differences detectable to females during mate choice has important evolutionary implications for how mtDNA may influence pre-copulatory sexual selection, thereby influencing the ultimate forces that shape genetic isolation between mtDNA haplotypes.

Specific design and methods

I will use genetic strains of *D. melanogaster*, created and maintained in my host lab, that each possess one of 13 different mtDNA haplotypes (collected from populations worldwide; Clancy 2008), but that share identical nuclear genomes (nuclear backgrounds). These strains offer a powerful way to partition the effects of genetic variation present in the mitochondrial genome from the effects of nuclear genome variation. However, without replication of these haplotypes across different nuclear backgrounds, it is impossible to separate mtDNA-specific effects from mito-nuclear interactions. The lack of replication at the mito-nuclear level has been a fundamental flaw in the methodology of previous studies that have tested for functional mtDNA variation.

As such, I am currently replicating these 26 strains (13 mtDNA haplotypes each kept in two independent populations) across three new isogenic nuclear backgrounds that have been maintained separately in my host lab. I will use a standard breeding scheme (Fig. 2A) to introgress focal mtDNA haplotypes into each target nuclear background. In total, I will establish 78 new *D. melanogaster* strains: 13 mtDNA haplotypes, each in two replicate populations, introduced into three nuclear backgrounds (Fig. 2B).

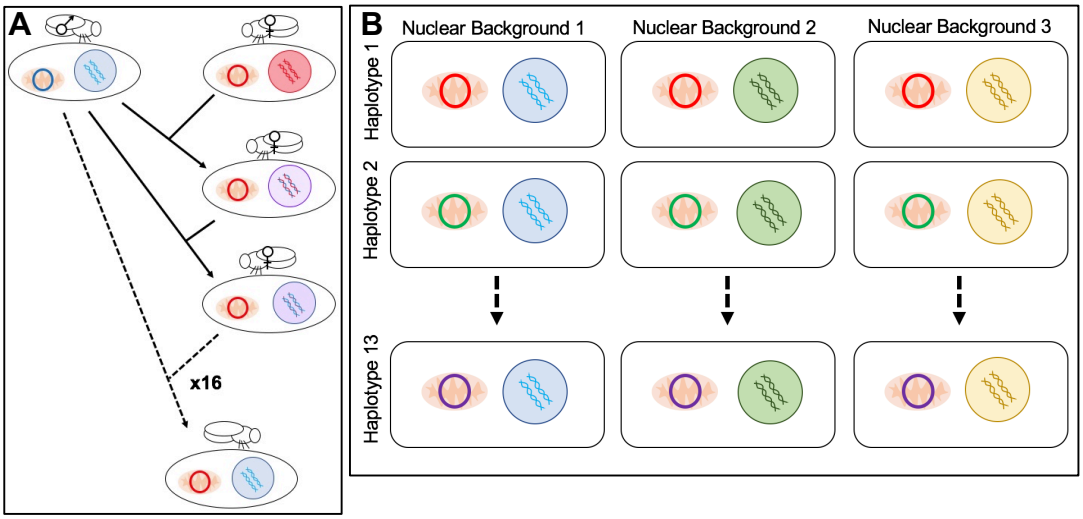


Figure 2. A) Crossing and backcrossing scheme to produce flies of the target nuclear background (blue) and mitochondrial haplotype (red). B) I will use back-crossing to generate 13 mtDNA haplotypes expressed in 3 nuclear genomes. Not pictured are the two experimental replicates of each mtDNA haplotype / nuclear background.

This opportunity to replicate the effects of mtDNA haplotype variation across multiple nuclear backgrounds is a key innovation that delivers unprecedented insights due to the inferential power of the strains and will establish a new resource for future research. The 13 haplotypes currently maintained in one standard nuclear background are the only such system in the world, and replicating these haplotypes across three new nuclear backgrounds will even further extend the novelty of this system. Further, the experiments I propose for my DECRA are only possible in this *Drosophila* system because each of the 78 strains I generate will exist as populations of genetically identical individuals (maintained through full-sibling crosses), which allows me to pool samples (e.g. small tissue samples) among individuals within a genetic strain without confounding results. Additionally, the nature of these strains enables me to compare the results of successive experiments on these strains, because individuals within a strain are genetically identical both within and among generations. This allows me unprecedented flexibility in my ability to draw generalized conclusions about specific mtDNA haplotypes from multiple, successive experiments.

OBJECTIVE 1: Determine how mtDNA haplotype affects the MOLECULAR phenotype through changes to mitochondrial efficiency and oxidative stress.

In this first experiment, I will characterize mitochondrial performance across mtDNA haplotypes in three tissues that are known to have high metabolic demands: the brain, the flight muscles, and the reproductive tissues.

Predictions: *Among haplotypes.* I predict that haplotypes will differ in performance across all three highly metabolically active tissues. These tissues are likely to experience any effects of small changes in mitochondrial performance mediated by variation in mtDNA.

Between sexes. I expect that mitochondrial function will be optimized for female rather than male performance, which will result in increased oxidative damage in the testes compared to the ovaries. In contrast, given that both

sexes require high functioning somatic tissue for survival (i.e. sensory and muscular function to locate and reach food sources), I expect no sex-specificity in mitochondrial performance in flight muscle or brain tissue.

Justification: A previous study on these 13 haplotypes of *D. melanogaster* (in one nuclear background) provided promising evidence to suggest sex-specific differences in mitochondrial characteristics across haplotypes, finding variation in mitochondrial density and respiratory rate (Wolff *et al.* 2016). Here, I build off this foundational study to better characterize specific measurements of mitochondrial quality and oxidative stress maintenance: mitochondrial efficiency, ROS production, and products of oxidative damage (collectively, "mitochondrial performance").

Mitochondrial respiration is a major source of reactive oxygen species (ROS) within the body, and these ROS—when produced at rates that outpace the cell's antioxidant defences—can cause harm by reacting haphazardly with cellular machinery (such as proteins or DNA) and disrupting function. Comparing the rate of oxygen consumed to the rate of ATP produced during mitochondrial respiration (P:O ratio) offers an estimate of mitochondrial efficiency because oxygen consumed *without* ATP generation has likely been used instead to produce ROS (Hinkle 2005; Figure 3). I will compare this estimate of mitochondrial efficiency with measures of cellular ROS levels and products of oxidative damage to compile three total measures for the overall performance of mitochondria of each mtDNA haplotype.

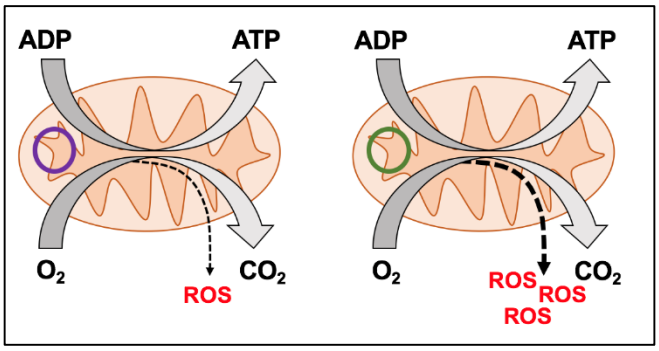


Figure 3. Mitochondrial produce ROS as a byproduct of cellular respiration. While it is important to signaling cellular conditions, high rates of ROS production can damage the cell as ROS react with and disrupt molecular machinery. Rates of ROS production depend both on rates of metabolism and on structural mitochondrial efficiency. I test whether mitochondrial haplotype (e.g. left or right, pictured here) may influence this latter source of ROS variation.

Measuring three different metrics related to oxidative stress (P:O, ROS levels, and oxidative damage) allows me a more detailed understanding of differences between tissues, sexes, and haplotypes. While all three measurements are fundamentally related, P:O offers an overall estimate of efficiency of ATP production, ROS levels indicate the specific output of those inefficiencies, and damage levels suggest whether the ROS produced is causing harm to cellular machinery (i.e. is not mitigated by antioxidant defences). While I may find these three measures to be tightly correlated, it is important to test for more complex interactions: it is possible that in some tissues, mitochondrial efficiency is low but oxidative damage measures are also low, which would indicate that inefficiencies are successfully prevented from causing functional damage. Such evidence of compensatory mechanisms for mitochondrial inefficiency is important to elucidate in order to better interpret higher-order physiological differences among haplotypes in the following experiments. These comprehensive methods for measuring mitochondria-related oxidative stress are another key innovation of this project.

Importantly, I measure three tissues with high metabolic activity to focus on a strong comparison of mitochondrial performance among individuals of different haplotypes or sexes; measuring a "control" tissue with comparatively low metabolic demand is neither necessary for the goals of this experiment, nor feasible: there is no data yet available on any tissues with low mitochondrial density and low metabolic demand in the fruit fly.

Specific methods: I will raise populations of the 78 experimental lines under standard maintenance conditions in density-controlled, mixed-sex vials. I will run each assay on mass-controlled, pooled samples of flies within the same experimental group; because these flies are genetic clones, measures of separate flies within one haplotype population can be confidently compared. Samples sizes in this experiment and in all further experiments have been designed based on power analyses, taking into account effect sizes from previous experiments finding haplotype- and sex-specific effects on phenotype (e.g. Camus *et al.* 2015).

I will sacrifice flies at 15 days of age, based on the age at which sex-specific differences in mitochondrial respiratory rate have previously been found across these haplotypes. After euthanasia, I will dissect out the thorax muscles, brain, and gonads (testes or ovaries) under a microscope using established techniques. For each method listed below, I will pool samples of each tissue from groups of 10 individuals for a total of 50 individuals per experimental replicate (mitochondrial haplotype, nuclear background, and sex), and I will homogenize tissues in media as described for *Drosophila* in Padalko (2005). My previous experience in these and other physiological measurements (Koch *et al.* 2018) will inform my approach to and interpretation of these assay.

P:O ratio. I will perform the P:O ratio measurement using methods described in Padalko (2005) and Salin *et al.* (2012). Briefly, I will incubate tissue homogenate in respiratory medium and measure oxygen consumption in a respirometer (OXYGRAPH-2K, Oroboros Instruments). Simultaneously, I will measure ATP production by adding

glucose and the enzyme hexokinase, which catalyzes the conversion of glucose into glucose-6-phosphate, converting one molecule of ATP back to ADP in the process (Figure 4A). I can then measure glucose-6-phosphate through fluorescence as a 1:1 indicator of the amount of ATP that is produced.

ROS measurement. I will quantify ROS levels in tissue homogenate using the DCFH-DA method, which uses a probe (DCFH) that fluoresces (as DCF) in the presence of intracellular ROS (Rhee *et al.* 2010; Figure 4B). This method has already been optimized for use in fruit flies within my host lab.

Oxidative damage. Finally, I will estimate oxidative damage in tissue homogenate by quantifying a stable product of oxidative damage to cellular proteins, protein carbonyls. I will use an established protocol (Protein Carbonyl Colorimetric Assay; Cayman Chemicals), which has been successfully used in *D. melanogaster* tissue (e.g. Rovenko *et al.* 2015). Briefly, I first isolate proteins from the tissue homogenate, then add the chemical 2,4-dinitrophenylhydrazine, which reacts with protein carbonyls to form a product that can be measured through color change.

After all three measurements have been collected, I will test for statistical correlations among the measurements within a tissue as well as among tissues. I will then will use mixed effects models to examine whether oxidative damage levels, P:O, and/or ROS levels depend in mtDNA haplotype, sex, or their interaction. A clear indicator of the presence of Mother's Curse mutations is much higher variance in performance among haplotypes in males than in females.

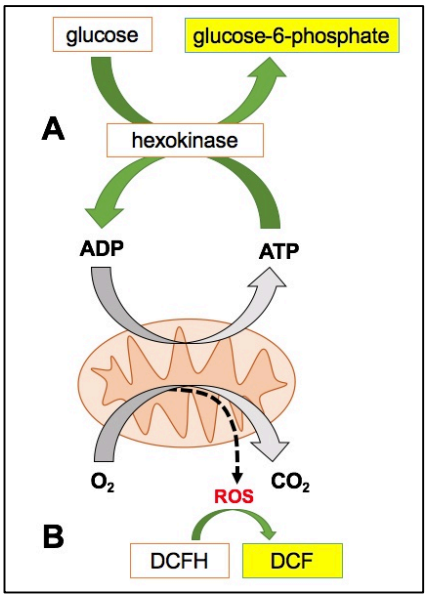


Figure 4. Methods of detecting ATP levels (A) and ROS levels (B). Yellow boxes represent products that can be measured through fluorescence.

OBJECTIVE 2: Evaluate how mtDNA haplotype alters individual performance in specific PHYSIOLOGICAL processes that have been linked to mitochondrial function.

I will test three major avenues of organismal physiology, each with strong connections to mitochondrial performance: innate immune defence, cognitive ability, and locomotor activity levels and performance.

Predictions: *Among haplotypes.:* I expect performance quality to vary across haplotypes, but the performance of specific measures within a haplotype to be positively correlated (positive pleiotropy), given that each measure relates back to fundamental aspects of mitochondrial performance.

Between sexes. I predict males and females to perform comparably on tests of innate immune system function and cognitive ability, as both sexes should benefit from improved immune defence and ability to make complex decisions regarding foraging and mate choice. However, *D. melanogaster* have already been discovered to face sexually antagonistic selection for locomotory *activity*: females with higher fitness are less active, while the opposite is true for males (Long & Rice 2007). Previous research has indeed revealed some variation in activity levels in males of different mtDNA haplotypes (Dean *et al.* 2015), providing a basis to expect activity level to vary with haplotype. Whether there is similar sexual conflict for locomotory *performance* remains untested, but it is possible that selection for highly active males and inactive females also imposes selection on their capacity to perform active behaviours. In sum, I predict sexual conflict to have induced Mother's Curse mutations detectable by greater variation in locomotory activity and performance among haplotypes in males than in females.

Justification: While there is considerable evidence from the biomedical literature that mitochondria are involved in each of these processes through energy production and/or signal mediation (Koch *et al.* 2017; Koch & Hill 2018), these relationships are often studied under conditions of severe clinical disease or in systems of mutants with major dysfunction. It remains an open question whether naturally occurring variation in the mtDNA sequence may cause phenotypic differences in variation in the performance of physiological processes, and whether these effects may differ between males and females.

In this experiment, I will build off of the foundational literature of mitochondrial involvement in physiological processes by testing whether mtDNA variation affects innate immune defences, cognitive ability, and locomotor performance. Each of these fundamental aspects of organism performance has been well studied (under different contexts) within *D. melanogaster*, so I can use established methods to test new questions of how mtDNA affects performance. I can then link the results of this experiment to those of Experiment 1 in the same lines of flies to search for connections between tissue-specific mitochondrial performance and physiological function (e.g. between flight muscle oxidative damage levels (Exp. 1) and flight endurance (Exp. 2) within a haplotype). Importantly, this experiment is not in any way contingent on the results of Objective 1: mitochondrial efficiency and oxidative stress (measured in the previous experiment) are only some of the many avenues by which mitochondria influence

physiological performance.

Specific methods: As above, I will raise populations of the 78 experimental lines under standard maintenance conditions in density-controlled, mixed-sex vials. Each of the tests will be performed on separate groups of 15-day-old flies, with 50 flies tested in total per experimental replicate.

Innate immune defence assay. I will test of innate immune defences by dosing flies with a live pathogen and measuring differential survival. In this experiment, I focus on anti-bacterial defences, a branch of innate immune defence with strong ties to mitochondrial performance (Cloonan & Choi 2013). I will use a standardized technique to dose males and females of the experimental lines with live *Micrococcus luteus*, a gram-positive bacterium that induces the Toll pathway of innate immune response and causes differential survival in *D. melanogaster* (Michel *et al.* 2001); *M. luteus* is classified as a risk group 1 microorganism (low risk) and will be isolated within my host lab's Physical Containment Level 2 laboratory. Briefly, I will dose individual flies under sterile conditions with a microinjection into the abdomen using a nano-injector (Nanoject, Drummond Scientific Company), as has previously been performed in my host lab. I will perform a dosage trial on a subset of non-experimental flies prior to commencing the main trials to determine the dose of *M. luteus* that induces approximately 50% survival in *D. melanogaster* within 48 hours, starting at a dose of 1 x 10^6 colony forming units suspended in PBS. I will maintain groups of 10 same-sex flies from each experimental replicate in vials of standard agar-based medium, tipping flies into fresh medium and recording the number of surviving flies every 24 hours for up to 96 hours.

Cognitive assay. I will test one aspect of cognition in *D. melanogaster* that has been used for more than ten years as a consistent estimate of associative learning and memory, important components of overall cognitive ability (Mery & Kawecki 2005). Such behaviours have relevance to foraging and mating success, and stimulate the development of neural pathways that may be costly: experimental evolution studies for increasing learning ability in *D. melanogaster* found decreased longevity in selected flies, particularly in males (Lagasse *et al.* 2012). These characteristics make this test a promising target in which to test for any possible sex- or haplotype-specific effects on a cognitive trait.

This assay has two phases: conditioning and testing. I will follow the protocol established and refined in Mery and Kawecki (2005) and subsequent studies. Briefly, I first will condition groups of *D. melanogaster* to associate a particular chemical odour (either 3-octanol or 4-methylcyclohexanol) with a mechanical "shock"—a one-second, 2000 rpm vibrational pulse from a test tube shaker. Within each experimental replicate, half of the flies will be conditioned to avoid 3-octanol, and the other to avoid 4-methylcyclohexanol. I will subject flies to two rounds of conditioning (odor exposure followed by mechanical shock), separated by 60 seconds of rest. After 24 hours, I will test the learning ability of flies by placing them, 10 at a time, in the center of a two-choice chamber, with 3-octanol venting through one end and 4-methylcyclohexanol through the other. The "learning ability" of the group can be quantified as the proportion of individuals that move away from the odor they learned to avoid during conditioning.

Locomotory assays. First, I will use a groundbreaking high-throughput phenotyping platform available in my host lab to assess activity levels of males and females of the experimental lines. This platform comprises six ZebraBoxes (ViewPoint Life Sciences), which are temperature- and light-controlled boxes illuminated under infrared and mounted with high resolution infrared cameras. The boxes can record fly movement and use software to extract target information, such as percent time spent walking or grooming. Each ZebraBox can measure up to 16 vials of 6 flies at once, each separated by an opaque barrier, allowing for a total of 96 flies to be measured simultaneously, and I will measure activity levels for 30 minutes.

Second, I will use the "negative geotaxis" test to evaluate the distance traveled during startle-induced climbing, a measure of climbing performance. In the negative geotaxis test, a vial containing flies is sharply tapped, causing all flies to drop to the bottom. The immediate behavioural response to *D. melanogaster* to such a drop is to begin a rapid climb up the wall of the vial; each fly's climbing performance can then be quantified as distance climbed in 4 seconds after drop. This measure is biologically relevant because it has been shown to vary with oxidative stress levels and decline with age (Jordan *et al.* 2012), parameters that relate to mitochondrial performance. To measure negative geotaxis in my proposed experiment, I will place same-sex groups of 10 *D. melanogaster* of each experimental replicate in vials containing standard agar-based food overnight for acclimation. Using racks that hold ten vials securely (Drosoflippers; drosoflipper.com), I can test ten vials of 10-individual populations simultaneously. After acclimation, I will stimulate the negative geotaxis behavior by sharply tapping the rack of vials once against a hard surface, and I will take a high-definition video recording against a clock and measurement scale to later use in measuring the average distance traveled by flies within 4 seconds.

Third, I will work with collaborator David Burton (Department of Mechanical and Aerospace Engineering, Monash University), the manager of the Monash Wind Tunnel, to test flight performance. Measuring flight in wind tunnels is prohibitive in most experiments due to lack of facilities, but the Monash Wind Tunnel Platform present at my host university offers an invaluable resource to test fly flight. Within the wind tunnel, I will release groups of 10 flies into

a custom-built apparatus based on the set-up used with *D. melanogaster* by Weber (1996): a 1.5 m by 12 cm by 12 cm tunnel with a bright light at the upstream end to stimulate flies to fly into the wind. This tunnel is divided into 40 equal-sized compartments, each with successively greater wind speed (regulated by pressure release valves in each compartment). Flies released at the dark, downstream end fly toward the bright, upstream end of the tunnel, facing progressively strong headwinds before reaching their limit and being blown backward (Weber 1996). I will use high-definition video cameras built-in to the wind tunnel apparatus to record flies' average maximum flight speed (the wind speed of the farthest compartment reached).

OBJECTIVE 3: Test how mtDNA haplotype affects BEHAVIOURAL performance and reproductive success.

I will test the quality of a central acoustic component to male *D. melanogaster* mating displays as well as the performance of males in mate choice trials with live females.

Predictions: *Among haplotypes.* I predict male *D. melanogaster* acoustic display quality will vary with mtDNA haplotype, which will translate to varying courtship success across haplotypes.

Justification: Previous study on these 13 mtDNA haplotypes have revealed haplotype-specific variation in male reproductive outcomes (Camus & Dowling 2017), but we do not yet know whether these effects are primarily mediated by pre-copulatory processes (such as courtship success) or post-copulatory processes (such as sperm competition). Male *D. melanogaster* perform a well-characterized and measurable courtship display to solicit copulations with females that allows me to test for the effects of pre-copulatory selection across haplotypes, extending the molecular and physiological data from Experiment 1 and 2 to behavioural data with tangible relevance to individual fitness.

The mating display of the fruit fly incorporates a mechanically produced sound (a "song" of pulsed tones from vibrating wings; Figure 5A) that has been shown to be involved in female mate selection, and males have been found to tactically adjust their courtship effort depending on previous experience with receptive or unreceptive females. The display involves aspects of cognition (learning tactics) and motor performance (wing vibration song), both of which are processes with considerable links to mitochondrial function (see above).

Sexual selection theory predicts that motor display traits like the wing vibration of male fruit flies often evolve to indicate some underlying aspect of male quality, such as his ability to sustain a prolonged muscular vibration or to produce vibrations of a particular style. Indeed, engaging in courtship behaviours appears to be costly for male fruit flies (Cordts & Partridge 1996). A key study of the display of *D. melanogaster* found that females preferred males with longer "pulse trains," which are sustained bouts of repeated, high intensity vibrational pulses that are presumably more energetically costly than the low intensity components of the song (Figure 5B; Talyn & Dowse 2004). These are exactly the types of display traits that may be sensitive to mitochondrial function, and sensitive to male-biased mutation accumulation through the Mother's Curse Hypothesis.

Because there is no direct female counterpart to the male mating display, I will not explicitly be testing for Mother's Curse effects. However, this experiment will be the first direct test of whether mtDNA variation influences male mating display performance, and the presence of significant variation in display quality among haplotypes will strongly suggest the accumulation of male-specific mutations. Such variation has important repercussions: if females can distinguish males by their mtDNA haplotype, this may provide a means of reinforcing haplotype-specific population boundaries through assortative mating, and may be a mechanism by which pre-copulatory mate choice could indirectly select against the accumulation of mtDNA mutations that harm male performance.

Specific methods: In this experiment, I will perform tests on virgin male *D. melanogaster* of the 78 focal populations, isolated in same-sex groups at the time of eclosion.

Acoustic quality of display. To quantify the acoustic properties of the pulse trains produced by males of different haplotypes, I will use an acoustic recording system built from the specifications of Arthur *et al.* (2013), which allows simultaneous recording and automated analysis of up to 32 males. I will place focal males with non-experimental, out-bred females (see below; to stimulate courtship song) in a small chamber paired with a sensitive pressure-gradient microphone (Knowles NR-23158). Each microphone is connected to the recording device via circuitry specifically designed to minimize noise and maximize recording quality for *D. melanogaster* courtship song. I will then use the open-source "FlySongSegmenter" software (Arthur *et al.* 2013) to automatically partition recordings from each male into component song parts, and analyse acoustic metrics important to mate choice, like inter-pulse interval (Figure 5B). This system is the only established array for high-throughput, high quality recording and analysis of *D. melanogaster* song, and is a key innovation that allows me to analyse the songs of focal males in a drastically reduced time frame relative to traditional measures (i.e. one pair with one microphone). I will measure the song of at least 20

males per experimental replicate.

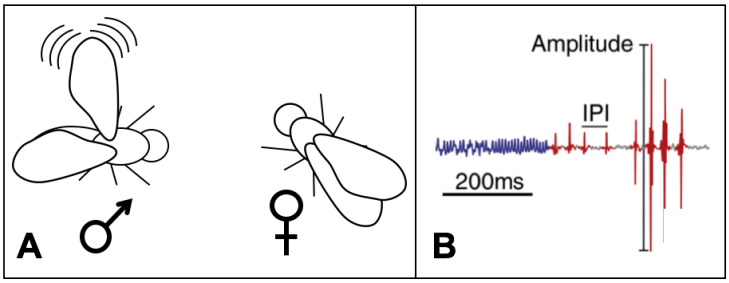


Figure 5. A schematic of the male *D. melanogaster* wing-vibration display (A), and a power spectrum of the acoustic portion of that display (B; from Coen & Murthy 2016). The power spectrum illustrates the "pulse train" (red) of high-amplitude, short vibrations. The inter-pulse interval, or IPI, is an important quality of the pulse train used in mate choice.

Courtship performance. To assess how variation in male display quality (including non-vocal traits) may translate to success in courting females, I will use the same high-throughput behavioural phenotyping platform described in Exp. 2, the ZebraBox, to track female mating preferences by measuring spatial association and latency to copulation. The platform, comprising six separate ZebraBoxes in my host lab, will allow me to video record the behaviour of up to 16 pairs of flies simultaneously, and the video output can be sent through software developed to track fly movement and record incidents of copulations. For each mate choice trial, I will release one virgin female and one virgin male *D. melanogaster* into 30 mm by 5 mm clear plastic tubes, separated with an opaque, removable separator at the midline. At the start of the trial, I will remove the separator to allow the pair to interact, and I will place the vial in recording position in a phenotyping platform. Each trial will run for 30 minutes, which is ample time for *D. melanogaster* pairs to reach copulation (average latency to copulate in this species is 19 minutes; Gowaty *et al.* 2003). I will optimize the software to automatically extract the time each female spends in close proximity (<2 mm), near (2-5 mm), and distant (>5 mm) to the focal male, as well as time where the male and female overlap (copulation). *D. melanogaster* courtship behaviour includes male chasing of females before approach and closer interaction (to receptive females only), so proximity of a focal male to the female is a good estimate of her interest. Latency to copulation is also noted as a key sign of female responsiveness.

To best isolate differences in female preferences for males of differing mtDNA haplotypes, I will test all males against out-bred females of the original populations that possess the three nuclear backgrounds used in created the mtDNA strains for this project (collected from Zimbabwe, New Guinea, and Chile; Clancy 2008). I will therefore test at least 10 males of each experimental replicate against three females, each from a different population. This volume of mate choice trials is only possible with the high-throughput behavioural phenotyping platform (the ZebraBoxes) available in my host lab, and offers unprecedented power to detect differences between mtDNA haplotypes. My experimental methods allow me to best isolate intersexual from intrasexual selection, and still allows for live interaction with visual, vocal, and chemical communication between individuals. At the end of the experiment, I can statistically quantify the time males of each replicate spent in close proximity to or copulating with females to estimate of female preference.

DECRA CANDIDATE

I will have no other roles or commitments and can dedicate 100% of my time to the proposed DECRA.

FEASIBILITY

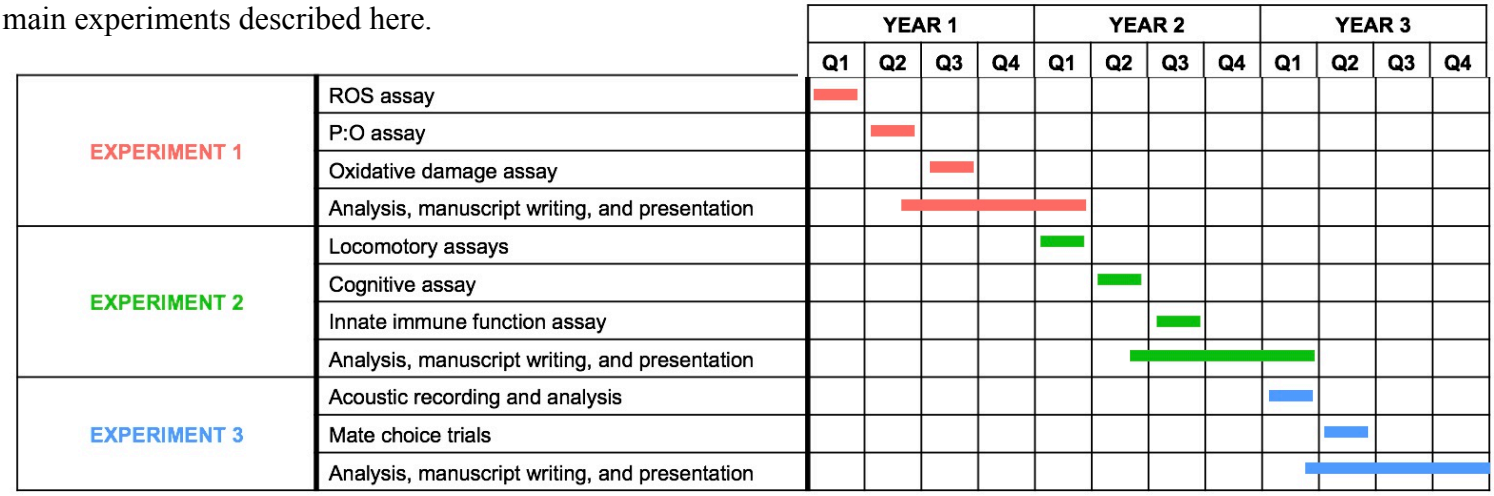
Experience of the DECRA candidate. Since joining my host lab in September 2017, I have gained the necessary skills in *D. melanogaster* maintenance, manipulation, and molecular analysis to complete this grant (e.g. ROS measurement, ZebraBox activity quantification). I have already initiated the line breeding to produce the 78 new mito-nuclear lines of *D. melanogaster* that I use in my proposed DECRA research, giving me a head-start to jump straight into experimentation should I receive funding. I have already analysed data from a large experiment of reproductive success in females of 13 different mtDNA haplotypes, and have written a manuscript for submission to a peer-reviewed journal (Koch, Phillips, Camus, & Dowling, in preparation for *J. of Evol. Biol.*). And, I have also initiated collaboration with David Burton (Monash University Wind Tunnel) to optimize the design of the *D. melanogaster* flight ability measurements.

In addition, my past experiences with animal physiology, behaviour, and mito-nuclear ecology have prepared me to complete this proposed project successfully. My recent work (Koch *et al.* 2018) involved a comprehensive suite of physiological measures incorporating aspects of innate immune function and oxidative stress measurement; many of these techniques have direct parallels to (or are even the same as) the measurements involved in Experiment 1 of my DECRA research. I also designed, measured, and analysed similar aspects of female mate choice for displaying males in captivity, including extracting measures of mating success from video (Koch & Hill, in preparation for *Anim. Behav.*). Further, my independent research project as an undergraduate focused on a mechanically produced sound in the male greater sage-grouse display (Koch *et al.* 2015), with surprising parallels to the *D. melanogaster* acoustic display: the key component of both sounds to mate choice is the time between high-intensity "pulses" (called the

"inter-pop interval" in sage-grouse, and the "inter-pulse interval" in fruit flies). My work with the sage-grouse has prepared me for the logistics of recording and analysing the sounds of male *D. melanogaster* in Experiment 3.

Lastly, I have a strong background in understanding the role of mitochondria in physiological processes. Indeed, I wrote the comprehensive references on two main topics I propose to examine in Experiment 2: how cognitive ability (Koch & Hill 2018) and immune system function (Koch *et al.* 2017) relate to mitochondrial performance.

Timeline. My proposed DECRA project is divided into three main experiments, and one year of my DECRA can be allocated to each. The new mito-nuclear *D. melanogaster* lines I am developing will be completed by November 2018, prior to the commencement of DECRA funding. I can therefore start Experiment 1 promptly. I will allocate three months to each of the sub-experiments of my DECRA (see table). This time frame—one year per experiment—gives me ample time to accommodate the large sample sizes of flies required to fully replicate tests across the 78 strains of flies I am examining, to present results at conferences, and to prepare manuscripts. The budget I have proposed will ensure sufficient funds to complete all three main experiments described here.



Research environment and facilities. The proposed DECRA project is only possible at Monash University, which has the collaborative research network and specialized facilities necessary for completion of the experiments I have designed. Monash University is consistently rated among the top 1% of universities worldwide, and is currently ranked 44th in the world for Biological Sciences by the 2018 QS World University Rankings. The School of Biological Sciences has benefitted from an $18.2 million investment in building and laboratory refurbishments since 2013, so I have access to state-of-the-art molecular and *Drosophila* facilities necessary for my proposed project. I am proposing to be hosted by A/Prof Damian Dowling, a world leader in mitochondrial evolution and genetics. His lab maintains the *D. melanogaster* strains necessary for my proposed experiments, and contains the high-throughput phenotyping platform (the six ZebraBoxes) for automated fly movement analysis—one of the only such platforms in the world. At Monash, I can foster collaborations with the Department of Mechanical and Aerospace Engineering to take advantage of the Monash Wind Tunnel as an exciting system for testing flight capacity in fruit flies. Monash University's School of Biological Sciences is also a powerhouse for evolutionary biology both inside and outside of *D. melanogaster*, and I will work closely—among others—with ecological physiologists Profs Craig White and Steven Chown and mitochondrial experts Dr Tim Connallon and Dr Matthew Hall.

This stimulating environment provides the stage on which I can further develop an independent record of high quality research and collaboration. The funding offered by DECRA will enable my financial independence in research, providing me greater autonomy in project design and execution. In addition, Monash University has a thriving community of post-graduate students and provides instruction in supervision, allowing me to attract a PhD student to work on projects associated with my DECRA research—and giving me the means to grow as an effective supervisor. The experiences enabled by DECRA will be invaluable as I forge my independence as a researcher and mentor.

BENEFIT AND COLLABORATION

Building new knowledge with innovative techniques. My proposed research lies at the cutting edge of evolutionary mitochondrial genetics. With the recent discovery that natural variation in mtDNA can have functional effects on phenotype, there is an urgent need to understand the consequences of these effects. Mitochondria are integral to a wide variety of physiological processes, from neural development to immune system function to fertility to aging. It is imperative that we understand how variation in the mitochondrial genome, so long thought to be inconsequential, can influence these important aspects of life. Adding a mitochondrial and mito-nuclear perspective to our understanding of nuclear genetic variation may finally offer answers to elusive questions about animal biology, including humans—such as why patterns of aging appear biased against males. My proposed DECRA project is not only a step, but a leap toward answering these questions. Such a comprehensive suite of molecular, physiological, and

behavioural measurements have never before been attempted on one system, and the system itself is unique in that it allows me to separate mitochondrial genetic and mito-nuclear effects. Each of the metrics I will measure in this system, from oxidative damage to mating success, offers a glimpse into massively complex processes; as such, my experiments will be the launching pad for a deep network of future study into mitochondrial genetics and physiology. By leading this research field, I hope to inspire a wave of new research that resonates across disciplines, thus augmenting Australia's research capacity in a field in which we are beginning to excel.

Cost-effective and valuable research. This proposed DECRA project makes clever use of existing resources already available at my proposed host lab and university, such as the wind tunnel and high-throughput phenotyping platform. My study system will already be developed at the start of my DECRA project, and the fast development and tractable husbandry of *D. melanogaster* make it an exceptionally cost-effective and valuable system in a host lab already optimized for fruit fly research. Most importantly, I have carefully selected each molecular, physiological, and behavioural measurement to maximize the power of the results—in terms of both statistical power and biological relevance—with minimal redundancy and cost.

Collaboration and education for national benefit. Understanding how mitochondrial and mito-nuclear genetics influence organismal health and fitness is an emerging research priority. My proposed research will build off the world-renowned research of my host lab to firmly situate Australia at the forefront of mitochondrial genetics, a field with cross-disciplinary relevance to evolutionary biology, biomedicine, and behavioural ecology. The results of this collaborative research program, integrating the expertise of a range of faculty at Monash University (including Profs Craig White, Moira O'Bryan, Paul Sunnucks) as well as an international expert in mito-nuclear ecology (Prof Geoffrey Hill, Auburn University, USA), will be of keen interest to both academic scientists and the greater Australian community alike due to its implications for understanding variation in human health and performance. My work will also have educational benefits through research and supervision of an associated PhD student, as well as supervision of honours students at Monash University. By engaging in the *Monash Research Supervisor Accreditation Program*, I will ensure that I am equipped to serve as a productive and supportive mentor. I will use my DECRA project as a platform to contribute to training Australia's next generation of scientists in cutting-edge research.

COMMUNICATION OF RESULTS

My host lab has had recent success publishing in high-impact journals (e.g. Innocenti *et al.* 2011, Reinhardt et al 2013), and the quality and broad scope of my proposed research will be well-suited for prominent journals like *Science*, *PNAS*, and *Current Biology*. I will present the results of my research annually at international scientific conferences, such as Evolution 2020 (Cleveland, OH, USA), and I will disseminate my results more broadly through Monash University's press releases and social media (Twitter, ResearchGate, personal website, Nature Ecology and Evolution Community).

MANAGEMENT OF DATA

I will upload all datasets and manuscripts to an online, open-access repository (figshare). Monash University has a specific repository within figshare to host these items under a Creative Commons license. This will allow any interested party to recreate my analyses from the original dataset, or re-use the data in future comparative studies or meta-analyses. I will also opt to publish open-access in peer-reviewed journals for at least one manuscript per year.

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