Challenges and Prospects for Testing the Mother's Curse Hypothesis

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Abstract

Maternal inheritance of mitochondrial DNA (mtDNA) renders selection blind to mutations whose effects are limited to males. Evolutionary theory predicts this will lead to the accumulation of a male-specific genetic load within the mitochondrial genomes of populations; that is a pool of mutations that negatively affects male, but not female, fitness components. This principle has been termed the Mother's Curse hypothesis. While the hypothesis has received some empirical support, its relevance to natural populations of metazoans remains unclear, and these ambiguities are compounded by the lack of a clear predictive framework for studies attempting to test Mother's Curse. Here, we seek to redress this by outlining the core predictions of the hypothesis, as well as the key features of the experimental designs that are required to enable direct testing of the predictions. Our goal is to provide a roadmap for future research seeking to elucidate the evolutionary significance of the Mother's Curse hypothesis.

The Mother's Curse hypothesis

In most species of bilaterian metazoans, the mitochondria (along with mitochondrial DNA, mtDNA) follow a strict mode of maternal inheritance; males never pass on their mtDNA haplotype to their offspring. This has intriguing evolutionary implications because it means that males must rely on females to screen the mtDNA sequence for new mutations—removing those that are deleterious and favouring those that increase fitness. Herein lies a catch. If a mutation was to arise that conferred harm to males only (a "male harming but female benign or near-benign" mutation), then selection would be blind to this mutation and it would be free to linger within the mitochondrial gene pool under mutation-selection balance (Frank & Hurst 1996). Moreover, if that mutation was actually sexually antagonistic in effect, harming males but benefitting females (a "male harming but female benefitting" mutation), then the mutation would be expected to be under positive selection due to its beneficial effect on females, and the population frequency of this mutation would be predicted to increase (Unckless & Herren 2009; Innocenti et al. 2011; Beekman et al. 2014). And in the converse scenario, if an mtDNA mutation arose that benefitted males at a cost to females, this mutation would be expected to be purged under purifying selection.

Maternal inheritance is thereby predicted to lead to the accumulation of mutations in the mitochondrial genome that depress male fitness but that are relatively benign or beneficial in females (Fig. 1). These population genetic principles, first discussed close to four decades ago (Cosmides & Tooby 1981) and modelled by Frank and Hurst in 1996, have come to be known as the "Mother's Curse" hypothesis, a term introduced by Gemmell and colleagues in a seminal publication in 2004. In the 15 years since this publication, interest has steadily increased in the hypothesis specifically (Unckless & Herren 2009; Wade & Brandvain 2009; Smith et al. 2010; Zhang et al. 2012; Beekman et al. 2014; Smith & Connallon 2017; Connallon et al. 2018), and the consequences of non-neutral genetic variation in the mitochondrial genome generally (Rand et al. 2004; Dowling et al. 2008; Burton & Barreto 2012; Ballard & Pichaud 2014; Dowling 2014b). This has led to a number of experimental and theoretical explorations of how Mother's Curse may (or

may not) underlie fundamental life-history patterns observed across taxa, such as the observation of females outliving males in many bilaterian metazoans (Tower 2006; Maklakov & Lummaa 2013; Dowling 2014a). However, despite firm theoretical foundations (Frank & Hurst 1996; Unckless & Herren 2009; Wade & Brandvain 2009; Hedrick 2011; Zhang et al. 2012; Smith & Connallon 2017; Connallon et al. 2018), the relevance of the Mother's Curse process to shaping patterns of phenotypic expression and the evolution of sex differences within natural populations remains controversial and unresolved (Beekman et al. 2014; Dobler et al. 2014; Eyre-Walker 2017; Vaught & Dowling 2018). Several factors have fed this controversy, including the lack of a clear predictive framework on which previous empirical studies of the hypothesis have been based, an absence of explicit tests of the hypothesis beyond more than one or two model systems, and a general lack of statistical power required to uncover Mother's Curse processes within natural populations if and when they exist.

The goal of our paper is to redress these factors by outlining a clear set of predictions upon which future tests of the Mother's Curse hypothesis should focus, and by developing a roadmap that may guide future research efforts in this field. We begin by addressing whether the theory developed in this field is compatible with processes of mitochondrial function that take place within living organisms. Specifically, we discuss whether mtDNA mutations that may confer sex differences in phenotypic expression have capacity to accumulate within natural populations. We then break down the foundational theory of the Mother's Curse hypothesis into two different forms, each of which yields its own predictions and experimental considerations. Finally, we consider how existing studies have tackled these predictions and the pitfalls they have faced, and how future research may better close the gap between the Mother's Curse hypothesis and the results we see in the real world.

Can Mother's Curse mutations manifest in the real world?

How might an mtDNA mutation have sex-specific phenotypic effects?

At first glance, it is difficult to envisage how a mutation within the mtDNA sequence could confer sex differences in its effects on organismal function (a "Mother's Curse mutation"). All of the genes encoded by the mitochondrial genome play central roles in eukaryotic life's most fundamental of processes: the production of ATP. Specifically, in bilaterian metazoans, the mitochondria encode 37 genes, 13 that encode subunits of key enzyme complexes of oxidative phosphorylation (OXPHOS), 22 that translate the mitochondrial mRNA into proteins, and 2 that encode rRNAs used to assemble the mitochondrial ribosomes (Blier et al. 2001). Assuming that the ATP requirements of males approximate those of females, one might thus conclude there is little or no capacity for sex-specific mutations to arise in the mtDNA sequence. That is, if a mutation were to arise in any of these genes that impaired OXPHOS function in females, that same mutation would be predicted to confer a similar impairment to OXPHOS function in males.

Yet, males and females present two very different environments in which the genome is expressed and functions. This is particularly evident in traits that exhibit high levels of sexual dimorphism or sex limitation—traits such as the gonads and gametes. The male gonad—the testis—is an engine of spermatogenesis, with high metabolic demands throughout adulthood (Short 1997). In humans, for example, each testis is capable of producing around 45 million sperm per day (Johnson et al. 1980). Each of these sperm contains a small number of mitochondria, typically between 50 and 75 (Ankel-Simons & Cummins 1996), which are thought to play a role in producing the ATP required to power their motility and to hence ensure their capacity for fertilization (Wu et al. 2019). In contrast, the ovaries and the eggs of females have very different characteristics to those of their male counterparts, and are likely to experience different metabolic requirements across the life course. The ovaries produce many fewer mature gametes over a lifetime, with the human female ovulating between 300 and 500 eggs (Derry 2006). Unlike gametogenesis in males, females produce all of

their eggs during juvenile ontogeny, which then remain in a prolonged state of metabolic quiescence prior to their ovulation in adulthood (Tosti & Menezo 2016). Furthermore, the copy number of mtDNA molecules per egg (~190,000 in humans) is vastly higher than the number in sperm (Reynier et al. 2001), which potentially reduces the metabolic burden on each individual mitochondrion. The constant turnover of gametes in the testes and the reliance of each individual sperm on a small number of mitochondria will plausibly render the spematozoa more sensitive to any small impairments to mitochondrial function than the ovaries and the eggs (Gemmell et al. 2004).

When considering these likely differences in the ontogenetic metabolic requirements of the testis and ovary, and the vast discrepancies in the mtDNA copy number of the sperm and ova, it becomes easier to reconcile how an mtDNA mutation might arise that confers a different effect on trait expression in each of the sexes. For example, an mtDNA mutation that has a clearly negative effect on the capacity of a sperm to swim quickly up the female reproductive tract may have a lesser effect, or no effect whatsoever, on the fertilization capacity of the egg and the subsequent growth of the zygote post-fertilization—particularly if the developing zygote was able to compensate for the effects of the mutation through increases in the copy number of mitochondria per tissue, as has been previously demonstrated (Pichaud et al. 2019).

Yet, as a consequence of the maternal inheritance of mitochondria, it is in the female environment—the ovaries and the eggs—that natural selection acts to screen the mtDNA sequence for variants that optimise gonadal and gamete function. Accordingly, it is conceivable that female-specific selection on the mtDNA sequence could then result in the selection of alleles that are optimised for female gonadal and gamete function, but possibly at the expense of male function. As such, maternal inheritance of mitochondria may lead to an inherent conflict between the sexes when it comes to which mitochondrial alleles are transmitted from one generation to the next.

Furthermore, while tissues tied to reproductive function arguably represent the most sexually dimorphic of traits (i.e. they are sex-limited), any trait that exhibits sex differences in its metabolic

requirements will likely experience sex-specific selection on mitochondrial function, and thus be a potential target for the accumulation of Mother's Curse mutations. Traits exhibiting such sex differences include other key components of adult life-history such as lifespan (Bonduriansky et al. 2008; Maklakov & Lummaa 2013; Dowling 2014a), as well as many behavioral traits associated with pre-copulatory success around breeding. For example, males may be more explorative or more combative than females (or vice versa). On the other hand, females may have higher metabolic demands associated with reproduction following copulation through disproportionate investment into the resourcing of offspring—from zygote to juvenile. Sex differences in behaviors performed over the duration of a season may place quite a different burden on the metabolic expenditure of males and females. Indeed, even the hormones often found to underlie many of these sex-specific behavioral patterns, such as testosterone, have key ties back to mitochondrial function; testosterone itself is synthesized within mitochondria, and steroid and thyroid hormones have been found to directly regulate the expression of nuclear genes affecting OXPHOS performance—and potentially mitochondrial genes themselves (Psarra & Sekeris 2008; Koch et al. 2017). Considering the different metabolic environments of males and females throughout the life course, be it through different hormonal profiles and behaviors or through fundamental differences in their reproductive organs, growth patterns, or morphologies, then the potential for mitochondrial mutations to confer sex differences in their phenotypic consequences becomes clearer. It is these sorts of sexually dimorphic traits that are predicted to be the key targets in which to test the predictions of Mother's Curse (Fig. 2). The examples we have described are, of course, not a complete list. We note for instance emerging evidence that several traits previously envisaged to be sexually monomorphic, such as those involved in organ development, gut physiology, general metabolism, and oxidative stress biology, are sexually dimorphic, providing scope for mtDNA mutations to affect their expression and performance differently in each of the sexes (Montooth et al. 2019).

Documented cases of Mother's Curse mutations

In plants, mtDNA mutations that impair the function of male components of reproductive function are widespread, conferring a phenomenon known as Cytoplasmic Male Sterility (Chase 2007). In contrast, it was long thought that similar cases of male-sterilising mtDNA mutations would not be found within the highly streamlined mtDNA sequences of bilaterian metazoans. Indeed, until recently there were no known examples of mtDNA mutations associated with male-limited fertility impairment in animals. However, several cases have emerged over the past decade. The clearest evidence to date for mtDNA mutations associated with Mother's Curse effects come from studies of reproductive traits in model species and humans (reviewed in Vaught and Dowling 2018). These include the identification of nonsynonymous mutations in three separate mitochondrial protein-coding genes (cytochrome B, cytochrome oxidase I and cytochrome oxidase II) in the fruit fly *Drosophila melanogaster*, which depress male fertility but have no clear negative effect on fertility of females (Xu et al. 2008; Clancy et al. 2011; Patel et al. 2016). Similar cases of mtDNA mutations associated with male-biased decreases in reproductive output have been reported in European hares (Smith et al. 2010), genetic strains of laboratory mice (Trifunovic et al. 2004; Nakada et al. 2006), and in humans with various forms of mitochondrial disease (Martikainen et al. 2017). Yet, reproductive traits are not the only targets for Mother's Curse type effects. Certain mtDNA mutations are known to cause a mitochondrial disease called Leber Hereditary Optical Neuropathy (LHON), which is associated with adult-onset blindness. The clinical penetrance of LHON is heavily skewed towards males—males represent around 80 percent of cases (Wallace et al. 1988; Man et al. 2002).

Remarkably, the identification of several of these documented cases of mtDNA mutations conferring Mother's Curse effects was serendipitous—their discovery was typically tangential to the main goals of the individual studies (Trifunovic et al. 2004; Nakada et al. 2006; Xu et al. 2008; Clancy et al. 2011). As such, few studies to date have explicitly sought to screen for Mother's Curse mutations (Vaught & Dowling 2018). Indeed, evolutionary theory proposes that many Mother's

Curse mutations are likely to be difficult to detect within populations because their very presence will have invoked strong selection on the standing pool of nuclear genetic variation of these populations for compensatory nuclear counter-adaptations, which rescue males from the negative effects associated with these mutations (Connallon et al. 2018).

A predictive framework for the Mother's Curse hypothesis

A key underpinning assumption

All formal empirical enquiry into the Mother's Curse hypothesis has been underscored by the implicit assumption that accumulation of Mother's Curse mutations will drive the evolution of nuclear compensatory adaptations. Each of the three predictions that we outline below extends on the assumption that many Mother's Curse mutations remain masked within populations, but can be unmasked if the mitochondrial haplotypes of these populations were to be placed alongside an evolutionarily novel background that lacks the requisite counter-adaptations required to offset the negative effects of these mutations. This underpinning assumption immediately places constraints on the types of model systems that are amenable to formal tests of the hypothesis, since the capacity to engineer strains of organisms that express precisely determined combinations of mtDNA haplotype and nuclear genetic background is generally limited to organisms that are experimentally tractable in the laboratory—those that are easy to rear, with short generation times (see Dowling et al. 2008 for an overview of how these strains are typically created). Accordingly, many of the formal tests of the Mother's Curse hypothesis have been performed in model invertebrate systems that allow for fine-scale manipulation of an organism's combined mitochondrial-nuclear (mito-nuclear) genotype. In particular, studies of *Drosophila* fruit flies have yielded promising evidence that different mtDNA haplotypes can affect males and females differently in traits like fertility and longevity, even at the small-scale level of variation present within a species (Camus et al. 2012; Camus et al. 2015; Camus & Dowling 2018). Such systems offer a powerful means to test

fundamental, proof-of-concept predictions of Mother's Curse. However, even in such tightly controlled systems, variation in experimental design can greatly alter how we interpret results in light of Mother's Curse, leading to discrepancies in both approach and conclusions in the literature. The goal of the following sections is therefore to develop a clear set of predictions and a unifying framework from which further study of the Mother's Curse hypothesis may progress.

The predictive framework

While the Mother's Curse hypothesis has attracted increased attention from empiricists over the past five years, the various tests of the Mother's Curse hypothesis to date have typically differed in the key predictions that they have sought to test. Clarification of the explicit predictions, and description of their nuances, strengths and limitations, is an important step needed to advance the field and to help in interpreting the findings of existing studies that vary both in their experimental designs and conclusions. Moreover, we posit that the Mother's Curse process must be partitioned into two forms, and we adopt the terminology of (Havird et al. 2019): a "weak form" originally envisaged by Frank and Hurst (1996), and a "strong form" based on the idea of direct sexual antagonism in the effects of mtDNA mutations. Here, we describe the basic predictions of these two forms of Mother's Curse, discuss their caveats and limitations, and consider the challenges faced in testing them.

The "weak form" of Mother's Curse

A population genetic model of Frank and Hurst (1996) shaped the conceptual development of this field by demonstrating that male-harming mtDNA mutations could be maintained under mutation-selection balance if the effects of these mutations were neutral or only slightly deleterious to females. This model describes a "weak form" of the Mother's Curse hypothesis, since these mutations are expected to accumulate only under processes of neutral evolution. That is, selection will be blind to mutations that are male harming but female benign, and this is predicted to lead to

the accumulation of a pool of mutations within the mitochondrial genome whose effects are felt only by males (a male-biased mitochondrial set of mutations, or genetic load). Furthermore, the size of this genetic load should differ across the mitochondrial haplotypes of different populations. This is because the different haplotypes will have evolved along their own trajectories, accumulating different numbers and severities of Mother's Curse mutations at different sites in the nucleotide sequence. In sum, natural selection should have removed mutations with negative effects on females, so haplotypes should converge in their effects on phenotypic trait values in females. But, selection will have left behind a male-biased genetic load in each haplotype, which will act to inflate levels of genetic variance across haplotypes for trait expression in males. As such, different haplotypes should confer differences in their effects on trait values in males. This leads to a simple quantitative genetic prediction.

Prediction 1: ***The genetic variation found across distinct mitochondrial haplotypes of any given species will confer larger effects on trait expression in males than in females.***

The methodological approach to testing this prediction is simple and follows in the footsteps of the classic quantitative genetic screens used to estimate levels of genetic variance attributable to different autosomal and sex chromosomes ("chromosome substitution" studies). The approach assumes that all parts of the genome are held constant with exception of the focal region under study (in this case, the mitochondrial genome). To achieve this, a researcher would a) sample a pool of mtDNA haplotypes from the spatial distribution of a given species, b) place the haplotypes alongside a standardized (and putatively "evolutionarily novel" nuclear background, with the intent of unmasking the pool of Mother's Curse mutations harbored within each of these haplotypes), and c) test the associated effects of these haplotypes on the expression of a range of focal phenotypes in each of the sexes (Fig. 3A, B).

This prediction arguably provides the most direct test of the Mother's Curse hypothesis, since it attempts to home in on the effects of mutational variation within the mitochondrial genome that are male-biased in magnitude. Researchers testing the Mother's Curse hypothesis in fruit flies (*D.*

melanogaster) adopted this approach by assembling a panel of thirteen mtDNA haplotypes sampled from disparate global populations and placing these alongside a single isogenic nuclear background (Clancy 2008; Camus et al. 2012; Wolff et al. 2016a). This panel has since been used to test the effects of the different mtDNA haplotypes on a range of phenotypic traits, from longevity to patterns of gene expression across the entire nuclear transcriptome, in each of the sexes (Innocenti et al. 2011; Camus et al. 2012; Camus et al. 2015; Wolff et al. 2016b). The results of these studies have generally provided strong support for this prediction of the weak form of Mother's Curse—variation in performance across haplotypes is typically larger in males than in females.

It is important to note that this approach does not involve researchers specifically documenting candidate Mother's Curse mutations, or even providing validation that the male-biased variation is deleterious in its action. In theory, mutations that are female-neutral but male-beneficial can also accumulate under the process modelled by Frank and Hurst (1996). However, the vast majority of non-neutral mutations that accumulate under processes of mutation accumulation (in the absence of selection) are expected to be deleterious in their effects (Orr 2010). This is expected to be particularly true for functional mutations that accumulate under the Mother's Curse process in the mitochondrial genome, given that these genes encode some of life's most important functions and evolve under strong purifying selection (Rand 2001).

The "strong form" of Mother's Curse

The "strong form" of Mother's Curse is so termed because it predicts that male-harming mutations will not merely accumulate within mtDNA, but instead will be selected for and thereby will increase rapidly in frequency through a population once originated. The key distinction between weak and strong Mother's Curse mutations is that the latter are directly sexually antagonistic: they boost female performance at the cost of male performance. If these mutations are predominant drivers of the Mother's Curse process, then in this case, we expect both males and females to exhibit variation in performance across mitochondrial haplotypes (Fig. 3D, E), but that the best-performing female haplotypes will be the worst-performing male haplotypes (Fig. 3F).

Given that Mother's Curse mutations have been predicted to be female-benign for much of the history of the theory (Frank & Hurst 1996; Gemmell et al. 2004), evidence for the "strong form" has only recently been uncovered (Camus & Dowling 2018). Yet, sexually antagonistic mutations have long been known to exist within the mitochondrial genomes of plants, and underlie the Cytoplasmic Male Sterility phenomenon. In many angiosperms, such mutations convert hermaphroditic plants into females—the plants lose their capacity to generate male gametes. This results in a breeding system known as gynodioecy. Female plants produce more seeds than their hermaphroditic counterparts, so these mtDNA mutations clearly augment female fitness at large costs to male components of reproduction (Budar 2003). Recently, a sexually antagonistic mutation has been described in *D. melanogaster*, located within the mitochondrial cytochrome b gene of respiratory complex III, which confers an amino acid transition (from alanine to threonine). This mutation, found within a haplotype that was originally sourced from Brownsville, Texas, is associated with decreased male fertility, ranging from mild reproductive impairment to full sterility across different nuclear backgrounds (Clancy et al. 2011; Yee et al. 2013; Dowling et al. 2015; Wolff et al. 2016c). Yet, females with this mutation do not suffer any clear reproductive costs, and young females actually appear to have *increased* reproductive success compared to their counterparts (Camus & Dowling 2018). The opposite pattern appears in tests of longevity: males carrying the Brownsville haplotype live longer lives than males with other haplotypes, while Brownsville females live shorter lives (Camus et al. 2015). Moreover, when it comes to juvenile components of fitness (egg-to-adult viability and pupal viability), individuals of both sexes that carry the Brownsville haplotype perform better than those of other haplotypes (Wolff et al. 2016c; Camus & Dowling 2018). These observations suggest this cytochrome b mutation will accumulate under direct positive selection within populations through the fitness benefits it confers to females and developing juveniles, despite its associated harm on adult males; this was recently substantiated by a study that tracked changes in frequency of this mutation across numerous experimental populations (Wolff et al. 2017).

These results suggest that sexual antagonism is both possible and present in the molecular architecture of mitochondrial genomes of metazoans. This leads to a clear prediction for the strong form of the Mother's Curse hypothesis.

Prediction 2: ***A negative intersexual genetic correlation will exist across haplotypes; haplotypes that are associated with high trait values in one sex will be associated with lower values in the other.***

The methodological approach to testing this prediction follows the same pipeline as Prediction 1: a large panel of haplotypes is created and placed against a standard nuclear background, then trait performance of each of the sexes is measured. Here, however, estimating the intersexual correlations across haplotypes is key (Fig. 3). As such, this same design can test Predictions 1 and 2 concurrently. Notably, these predictions are not mutually exclusive, and a given panel of populations that vary in mtDNA sequence may exhibit a mix of mutations that alter male performance, female performance, or both—and such results may additionally vary among traits. On one hand, this makes it difficult to separate the relative contributions of both the weak and strong forms of the hypothesis to the Mother's Curse process since the effects of just a few "strong form" mutations may hide the effects of many "weak form" counterparts. Yet, on the other hand, making the distinction between weak and strong forms is important because unless the data is visualized and tested from both perspectives, Mother's Curse effects that are present across a panel of haplotypes may not be detected, and thus erroneous inferences deduced. For example, Camus and Dowling (2018) studied various components of reproductive performance in male and female *D. melanogaster* using the same panel of 13 mitochondrial genotypes described above. Previous studies from the same lab have published evidence suggestive of the weak form of Mother's Curse mutations in this panel affecting longevity—evidence that mtDNA variation causes phenotypic variation in males but not females (Camus et al. 2012). Camus and Dowling (2018) found no such pattern in their measurements of reproductive success (Fig. 3 D, E). Only after examining

intersexual correlations in performance did the strong form of Mother's Curse become apparent (Fig. 3 F).

Tests for nuclear compensation of Mother's Curse effects

As outlined above, the tests of all predictions of the Mother's Curse hypothesis assume that nuclear counter-adaptations evolve that offset the effects of the Mother's Curse mutations, and thus a goal of such tests is to first place a set of focal mtDNA haplotypes alongside a novel nuclear genetic background to which the mtDNA haplotypes have not directly coevolved. Yet, whereas tests of Predictions 1 and 2 place the haplotypes against a common nuclear background and then seek to directly estimate the effects of mitochondrial variation on phenotypic expression in each of the sexes, a third set of tests has emerged that hinge on a separate standalone prediction.

Prediction 3. ***Experimental disruption of putatively coevolved combinations of mitochondrial and nuclear genotype will lead to decreases in fitness, with the magnitude of the fitness loss being greater in males.***

This prediction is founded first on evolutionary predictions that posit the disruption of coevolved pairings of mito-nuclear genotype will lead to general reductions in fitness in both of the sexes (i.e., in ways independent of sex-specific mutation accumulation), since tightly coadapted pairings are no longer expressed together in the disrupted form (Rand et al. 2004; Wolff et al. 2014). This principle has been compellingly demonstrated by empirical work on the splash-pool copepod, *Tigriopus californicus* (Ellison & Burton 2008). Prediction 3 extends this assumption to posit that males will exhibit greater decreases in performance than do females, because in addition to the negative consequences of disrupting tightly coevolved mito-nuclear gene pairings, males will suffer the consequences that their Mother's Curse mutations are no longer masked by compensatory nuclear mutations (Fig. 4).

Studies addressing this prediction thus seek to test for the presence of effective nuclear compensatory adaptations that rescue populations from the effects of Mother's Curse mutations.

Perhaps the clearest evidence of this nuclear rescue effect to date comes from a study of *Drosophila* in which (Sackton et al. 2003) found that the activity of cytochrome c oxidase (a key mitochondrial enzyme) was significantly disrupted by interspecific hybridization—placing the mtDNA of *D. simulans* into the nuclear background of *D. mauritiana*—in males, but not females. Little other evidence exists for this prediction currently, and indeed, other studies to test it so far have not found consistent signatures of male-bias in the magnitude of fitness loss in each of the sexes during similar cases of hybridization (Immonen et al. 2016; Mossman et al. 2016a; Đorđević et al. 2017). For example, in a study of the prediction in the seed beetle *Callosobruchus maculatus*, Immonen et al. (2016) found that disruption of population matched mito-nuclear genotypes led to decreased lifetime fecundity in females, but no discernible effects on lifetime reproductive performance in males. And, Đorđević *et al.* (2017) reported general decreases in mitochondrial electron transport chain activity in both sexes when disrupting the mito-nuclear combinations of populations of the seed beetle *Acanthoscelides obtectus* that had been selected for short or long life, albeit some disrupted combinations suffered male-biased decreases in longevity, consistent with prediction.

We also note that it is possible that Mother's Curse mutations will exist within a population but not be effectively offset by counter-adaptations. Indeed, the "Brownsville haplotype" described above has been found to impair male fertility across a wide range of nuclear backgrounds (Dowling et al. 2015), and even 10 generations of experimental evolution (in large laboratory populations with high levels of standing nuclear variation) failed to prompt the appearance of a compensatory nuclear mutation (Wolff et al. 2017). Testing Prediction 3 therefore is important not only to validate generality by which nuclear compensation is a viable mechanism by which males may circumvent the harmful effects of Mother's Curse mutations, but could in theory lead to the discovery of new cases of Mother's Curse mutations for which effective nuclear rescue apparently does not occur.

Inferential and methodological considerations: a roadmap for future studies

The growth in the number of studies seeking to test these three predictions of the Mother's Curse hypothesis has been encouraging (Innocenti et al. 2011; Immonen et al. 2016; Mossman et al. 2016a; Mossman et al. 2016b; Đorđević et al. 2017; Camus & Dowling 2018), although we believe the research efforts to establish the broader generality of the Mother's Curse hypothesis to natural populations are currently in their infancy. Below, we outline some methodological limitations of previous studies to test this hypothesis, and discuss a roadmap for future research.

Three key levels of replication for future studies

Robust tests of each of the three predictions, going forward, will hinge on adequate replication at three levels: a) the number of mitochondrial haplotypes sampled, b) the number of nuclear backgrounds in which these mitochondrial haplotypes are tested, and c) the individual genotype (each genotypic combination should be independently replicated within a panel of strains).

Replication of mitochondrial haplotypes. The goal of tests of Predictions 1 and 2 is to partition patterns of genetic variation in the focal genomic region from other confounding sources of variance, such as variation in other parts of the genome or environmental sources of variation. When sampling genotypes from a natural pool of variation, studies that hinge on quantitative genetic assumptions should attempt to sample a reasonable fraction of the genetic variation that exists in nature to avoid the effects of sampling bias and to increase statistical power (Fig. 5). Failure to do so means that inferences of the studies may only be relevant to the particular haplotypes sampled in those studies. For instance, some studies have sought to make inferences as to the generality of the Mother's Curse process in their populations when comparing sex specificity of effects across two haplotypes (Mossman et al. 2016b; Aw et al. 2017). While the results of these studies have been intriguing (Aw et al. found the predicted males bias, while Mossman et al. reported a strong female-bias contrary to Prediction 1), some caution should be applied to

inferences seeking to extrapolate these results beyond the specific haplotypes studied. Rather, these studies provide proof-of-concept insights and motivation on which to further investigate the effects across a broader pool of haplotypes. This issue of replication extends to tests of Prediction 3—sampling bias exists whenever the independent unit of replication (the number of contrasts between matched and mismatched mito-nuclear combinations) is low.

Replication of nuclear backgrounds. As described above, many of the studies that have explicitly sought to test Predictions 1 and 2 of the Mother's Curse hypothesis have developed systems that place the focal genomic region under study (mtDNA) against a highly controlled genomic background (Innocenti et al. 2011; Camus et al. 2012; Wolff et al. 2016b; Camus & Dowling 2018). These studies have provided important evidence for the Mother's Curse hypothesis. This approach, however, raises an important design consideration. It is increasingly clear that mitochondrial function hinges on interactions between proteins encoded by both mitochondrial and nuclear genomes, and thus we should expect that the link between mitochondrial haplotype and phenotype is moderated at least to some degree by nuclear background (epistatic mitonuclear interactions). Indeed, there is strong evidence that such interactions are key determinants of phenotypic expression (Arnqvist et al. 2010; Dowling et al. 2010; Zhu et al. 2014; Mossman et al. 2016a). This raises an important question: would previous patterns of male-bias in the magnitude of mitochondrial haplotype effects, consistent with Prediction 1, be upheld if the same haplotypes had been sampled in an alternative nuclear background? Future work should strive to test these same predictions against a variety of different isogenic nuclear backgrounds to determine whether the patterns of male-bias or sexual antagonism revealed against isogenic backgrounds used previously are upheld across at least some other nuclear backgrounds.

The issue of balancing the number of mitochondrial haplotypes sampled against the number of nuclear backgrounds is a difficult issue to resolve, given the logistical constraints inherent to studying genetic designs involving many experimental units—the number of which can quickly expand beyond a lab's ability to study them. Sampling every possible mitochondrial haplotype or

nuclear background is impossible, and researchers must inherently sacrifice replication at some level in order to complete experiments. However, we caution researchers against reducing the number of mitochondrial haplotypes (since this is the genomic region of focal importance for inferences of Predictions 1 and 2) in order to increase the number of nuclear backgrounds. Yet, another problem arises: if sampling just a few nuclear backgrounds, then inferences might be obscured by sampling bias in the nuclear background—given the near infinite number of nuclear backgrounds, this issue is a difficult one to solve. For example, what if Predictions 1 and 2 were upheld across just 1 of 3 nuclear backgrounds included in a study, while in the real world, they would be upheld across 75% of nuclear backgrounds—or across just 5%?

One possible way around this problem would be to translocate the panel of haplotypes into replicate mass-bred populations of the study species, such that each haplotype is expressed alongside a representative pool of nuclear variation captured from the one large and panmictic outbred population. The limitation of this approach is that all of the segregating nuclear variation within a strain will likely swamp the effects attributable to the mtDNA haplotype, making it difficult to partition the mitochondrial variance effectively. Furthermore, these pools of nuclear variance will quickly diverge across strains, which would completely confound estimates of mitochondrial variance. To overcome these limitations, one would need to create numerous replicates of each mitochondrial strain, and backcross females of each strain to the source population to attempt to prevent divergence of the nuclear genomic background. An alternative approach would be to run the experiments in two stages: a) use the approach previously leveraged to test many mitochondrial haplotypes against a single isogenic background, use the results of this first test to focus in on a subsample of haplotypes exhibiting the highest levels of sex difference in trait expression, then b) create and test a reduced panel of these haplotypes expressed alongside a larger number of isogenic nuclear backgrounds.

Clearly, the issues of replication of each of the focal mitochondrial genomic regions and their nuclear backgrounds are difficult to balance. Nonetheless, we urge researchers to maximize

replication in their experimental lines across multiple axes by sampling mitochondrial haplotypes and nuclear backgrounds broadly.

Replication at the level of the genotype. Furthermore, numerous studies that have sought to test the dynamics of mito-nuclear interactions, or the Mother's Curse hypothesis *per se*, have been limited by a lack of independent replication of the statistical unit of inference—either the mitochondrial haplotype or mito-nuclear genotype. This limitation affects the inferential power of these studies, since the lack of this level of replication renders it technically impossible to statistically partition true mitochondrial genotypic effects (or effects of mito-nuclear mismatching) from confounding sources of variation. This confounding variance includes effects attributable to nuclear genotypic differences that will invariably accrue and diverge exist across the panels of mitochondrial or mito-nuclear strains under study, as well as confounding effects of environmental variance such as the effects of shared environments (individuals of a given genotype all typically share the same environment—stored within the same enclosures). The effects of these confounding sources of variance on phenotypic trait values may be large relative to the expected effects of the mitochondrial genotypes; for instance, even a very small amount of cryptic nuclear variation that accumulates across a set of mitochondrial strains may exceed the genetic variation that exists across the focal haplotypes. It is therefore important not only to work to reduce the effects of sampling bias by testing a wide range of mitochondrial haplotypes and nuclear backgrounds, but also to create and test independent replicates of the focal genotypes to separate the genetic effects of interest from unintended sources of variation.

Which traits to measure

As outlined above, evolutionary logic would predict that sensitivity of any given trait to the Mother's Curse process will increase with increasing sexual dimorphism of the trait (Fig. 2). When it comes to mtDNA-mediated optimisation of mitochondrial function of any given sexually

monomorphic trait, males should salvage the benefits of selection on the mtDNA for optimised function in the female homolog of this trait. These benefits erode for sexually dimorphic traits, and in particular for adult life history traits in which the magnitude and direction of selection typically differ across the sexes. These are the traits that are the most promising candidates to reveal Mother's Curse effects. Such traits may be those most difficult to measure accurately in both sexes, as highly dimorphic traits inherently take quite different forms between the two sexes. For example, studies in egg-laying species may assess female reproductive success by counting numbers of eggs produced (fecundity), size of the eggs (investment per gamete), or proportion of eggs hatched (fertility); in contrast, measurements of male reproductive success may comprise quantity and viability of sperm, or success in acquiring copulations or producing viable adult offspring. Such traits are not direct analogues of each other (i.e. quantity of sperm produced by a male is quite separate in form and function from number of eggs produced by a female), but we argue that these traits are nevertheless exactly where Mother's Curse effects are most likely to manifest. If a trait is all-but-identical in form between males and females, then there is little basis for mutations to affect males and females differently—the fundamental premise of Mother's Curse. It is instead important to focus on comparing traits that are analogues in function or fitness consequences, such as traits that ultimately underlie "reproductive success."

Furthermore, it is now clear that mitochondrial polymorphisms can routinely exert complex patterns of negative pleiotropy on different traits, both within and between the sexes. The cytochrome-b mutation harboured within the Brownsville haplotype described above is one such example that serves as an excellent case for the importance of measuring multiple fitness-related traits. Had the authors of these studies only examined longevity effects associated with their panel of mtDNA haplotypes, they would have concluded that the effects of the Brownsville haplotype are opposite to those predicted under the Mother's Curse hypothesis—female-harming but male-beneficial (Camus et al. 2012; Camus et al. 2015). Had the authors measured only juvenile components of fitness (egg-to-adult viability and pupal viability), they would have concluded that the mutation that delineates

the Brownsville haplotype is adaptive to both sexes (Wolff et al. 2016c; Camus & Dowling 2018). However, the large costs to adult male infertility outweigh the modest lifespan extension afforded to males by this mutation (Clancy et al. 2011; Yee et al. 2013; Camus & Dowling 2018), and clearly it is therefore representative of a classic Mother's Curse mutation.

In light of these cautionary notes, we advise that researchers focus on the measurement of several components of life-history across the life-course in order to fully understand the patterns of sex-specificity across diverse mtDNA haplotypes. However, inferences as to whether the patterns concord to predictions of Mother's Curse must take into account the relationship of the measured traits with sex-specific fitness outcomes. As we have mentioned above, the most promising targets of the Mother's Curse process are those traits that are sexually dimorphic and in which the direction and magnitude of selection is known to diverge across the sexes.

Biological scale

Finally, it is important to consider the implications of variation in biological scale when testing the predictions of the Mother's Curse hypothesis. Several studies have sought to apply the key predictions of the Mother's Curse hypothesis to inter-species comparisons of mito-nuclear combinations. They have done so, for instance, by placing the mtDNA haplotypes of congeneric species alongside the nuclear background of one of the two species, and then interpreting patterns of sex-specificity in effects in the context of the predictions of Mother's Curse (e.g., (Mossman et al. 2016a; Mossman et al. 2016b)). Such an approach is appealing because it maximises divergence between the mitochondrial genomes under comparison, thus presumably increasing the opportunity by which mito-nuclear incompatibilities may be revealed upon inter-specific crosses.

However, a body of theory on phenotypic plasticity under extreme environments suggests that placing a set of genotypes into a highly novel environment to which those genotypes have had no prior history of selection could well expose cryptic, but nonadaptive, genetic variation (Chevin &

Hoffmann 2017). Accordingly, placing the mtDNA haplotype of one species against the nuclear background of a separate and evolutionary divergent species (which exhibits no natural occurrence of introgression) may then be akin to placing the mtDNA into an extreme selective environment. This could render mtDNA polymorphisms, which were honed by selection (and were thus adaptive) within the nuclear environment of the species in which they evolved, no longer adaptive within the divergent nuclear environments of the foreign species. In the context of the Mother's Curse hypothesis, this raises the non-trivial possibility that mtDNA mutations that are female-benign (or beneficial) but male-harming when placed alongside the pool of nuclear backgrounds of the species in which they evolved, may no longer exhibit the same fitness effects in females and males when placed alongside putatively-extreme nuclear backgrounds of a different species. The reaction norms associated with particular mtDNA mutations or haplotypes in the new nuclear environment may well be nonadaptive.

Put simply, Mother's Curse mutations may no longer act like Mother's Curse mutations in the new and extreme nuclear context, and if so, this would obscure the capacity with which to test the predictions of Mother's Curse within an inter-specific context. As a case in point, some mtDNA mutations that confer mitochondrial disease in humans, and which segregate at low frequencies within human populations, appear to be fixed in other lineages of some of our closest hominid relatives (de Magalhaes 2005; Queen et al. 2017; Tavares & Seuanez 2017). This includes two separate mutations in mt:ND1 that are associated with LHON in humans (a mitochondrial disease exhibiting high male-biases in penetrance). One of these mutations (A132T) is confirmed to cause LHON in humans, but is present in the reference sequence of the Bornean orangutan, *Pongo pygmaeus,* and sooty mangabey, *Cercocebus atys*. The other mutation (A64S) appears to be fixed amongst closely related hominids (orangutans, gorillas and chimpanzees) (Tavares & Seuanez 2017). The implication here is that there are mutations that appear to be pathological in the nuclear contexts of humans, but might be adaptive or neutral in the nuclear backgrounds of other species, including other hominids. In other words, putative LHON-causing mtDNA mutations appear in the

reference sequences of other hominids, with no records that they cause disease in these other species. This example provides some insight into potential caveats of tests that take the inter-specific approach. We therefore urge researchers to carefully consider issues of biological scale when interpreting phenotypic effects, resulting from inter-species mix-and-matching of mito-nuclear genotypes, in the context of the predictions of the Mother's Curse hypothesis.

Conclusion

The studies of the Mother's Curse hypothesis conducted to date have provided important proof-of-concept on which to base a roadmap for future study. These studies have provided valuable insights, but have also revealed the complexity in both the predictions and inferences that come from tests of this hypothesis. We urge that studies of large panels of mtDNA variation begin to validate previously-reported male-biases in the magnitude of mitochondrial genotypic effects in other nuclear backgrounds (captured from the same species). This is difficult given that emphasis needs to be placed on having an adequate representation and replication of mitochondrial genotypes across more than one nuclear background, which will present challenging logistical constraints. To resolve this, one may select key haplotypes that previous tests have shown to be associated with large degree of male bias (weak form) or sexual antagonism (strong form), and take a targeted approach to testing the sex specific effects of these haplotypes across a large number of conditions. Furthermore, future studies should redress issues pertaining to levels of replication and biological scale explicitly in their experimental designs, or otherwise ensure caution when interpreting results in light of these considerations. Studies should also move beyond the core model species of *Drosophila* to incorporate other systems that are experimentally tractable, and to natural populations where applicable and appropriate.

In conclusion, the controversies surrounding the generality of the Mother's Curse process in nature are not fully resolved, but their resolution has been hindered by a lack of a clear predictive

framework on which to design experimental tests of the hypothesis. Our goal is that the concepts discussed in this paper inspire others to turn their attention to these predictions, and to expedite a resolution to this outstanding question in the field of evolutionary biology.

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Figure Captions

Figure 1. The evolutionary mechanism underlying Mother's Curse can be visualized as a "sex-specific selective sieve" (Innocenti et al. 2011). Natural selection can be thought of as a sieve that filters out harmful mutations, preventing their spread through the next generation. However, because males do not pass mitochondrial DNA on to offspring, there is no means by which natural selection can act to remove male-harming (but female-neutral or beneficial) mitochondrial mutations from the population. As such, mutations that harm females (red circles) are selected out, but mutations that affect only males (black triangles) can spread throughout a population.

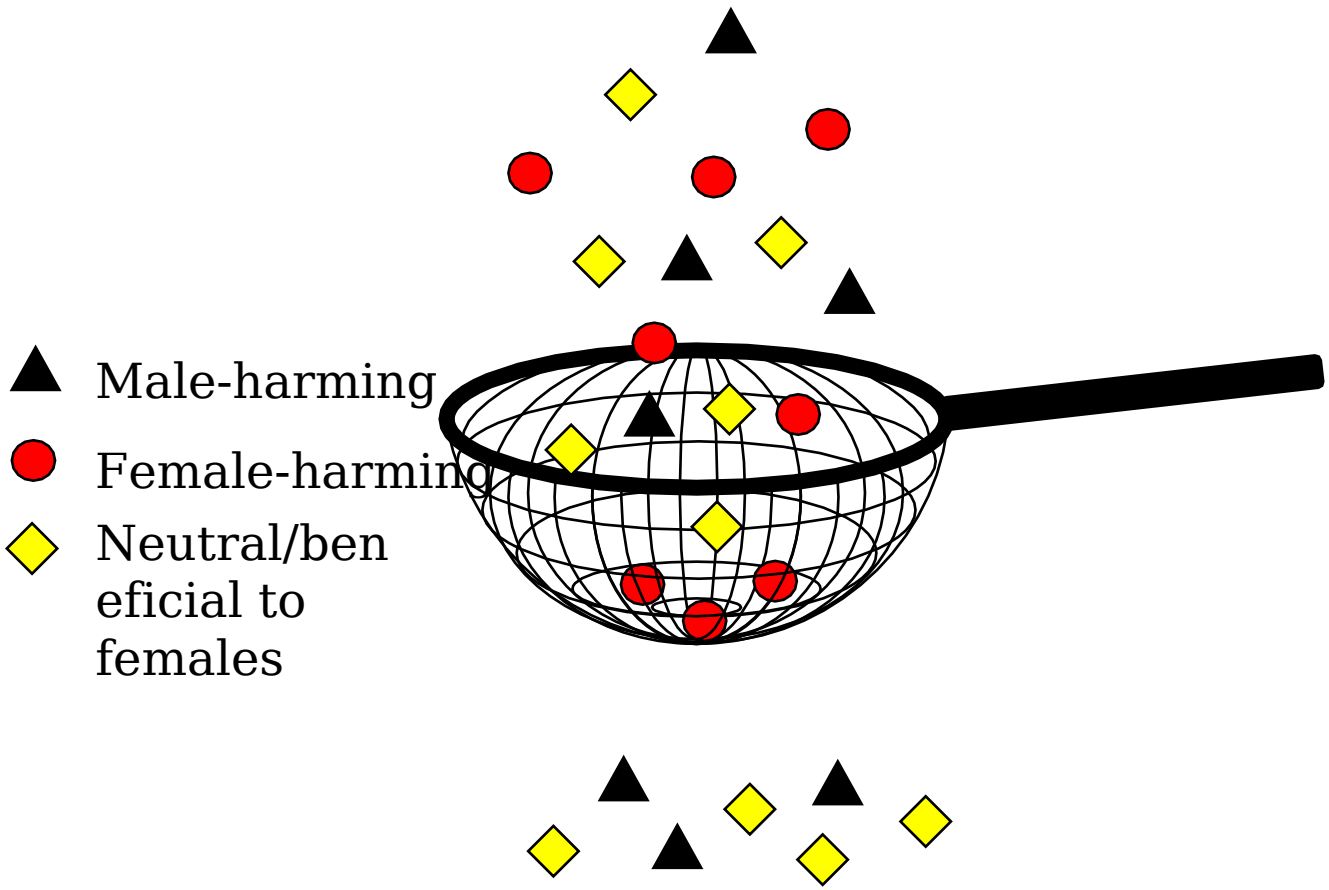
Figure 2. The capacity for mtDNA mutations to exert sex-specific effects on any given trait is predicted to increase with the level of sexual dimorphism separating the female and male homologs of the trait. Traits with greater sexual dimorphism are more likely to pose different metabolic environments in males than females, increasing the chance that mtDNA mutations will have different (or perhaps opposing) effects between the sexes. Thus, the potential for Mother's Curse to affect the expression of any given trait should scale with the level of sexual dimorphism; from low potential in sexually monomorphic traits, to high potential in sex-limited traits.

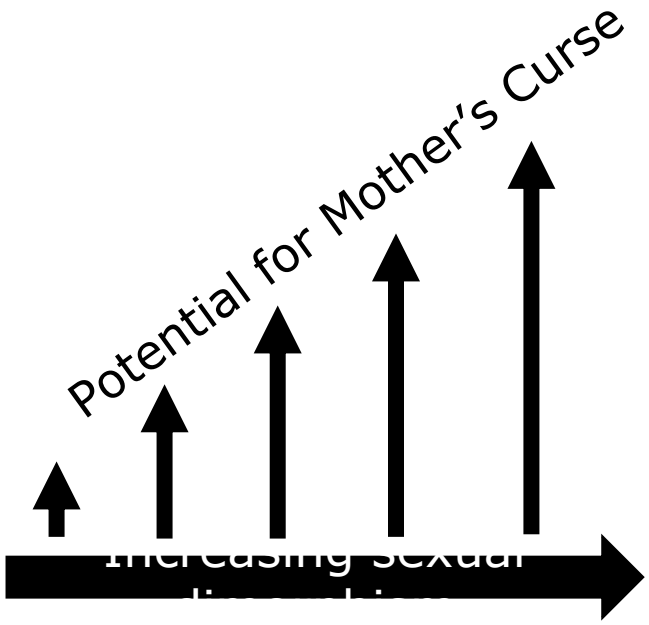
Figure 3. The top three panels (A-C) demonstrate results that are expected under the weak form of Mother's Curse (adapted from unpublished data). Here, males (A) show greater variation in performance among haplotypes than do females (B), but there is no clear evidence of sexual antagonism (C). In contrast, the bottom three panels (D-F) demonstrate results that are expected under the strong form of Mother's Curse (adapted from published data; Camus and Dowling 2018). Males (D) and females (E) both show variation in performance among haplotypes, but the mutations distinguishing these haplotypes appear to be sexually antagonistic in nature (F); the best-performing haplotypes for females are the worst-performing haplotypes for males. In panels A, B, D, and E, the solid horizontal line represents the mean, and vertical dashed lines illustrate that haplotype's variation from the mean. The solid blue line in panel F indicates the significant negative correlation between male and female performance across these lines. Note that all haplotypes represented here are expressed with one isogenic nuclear background such that only mitochondrial genetic variation is expected to influence variation in performance.

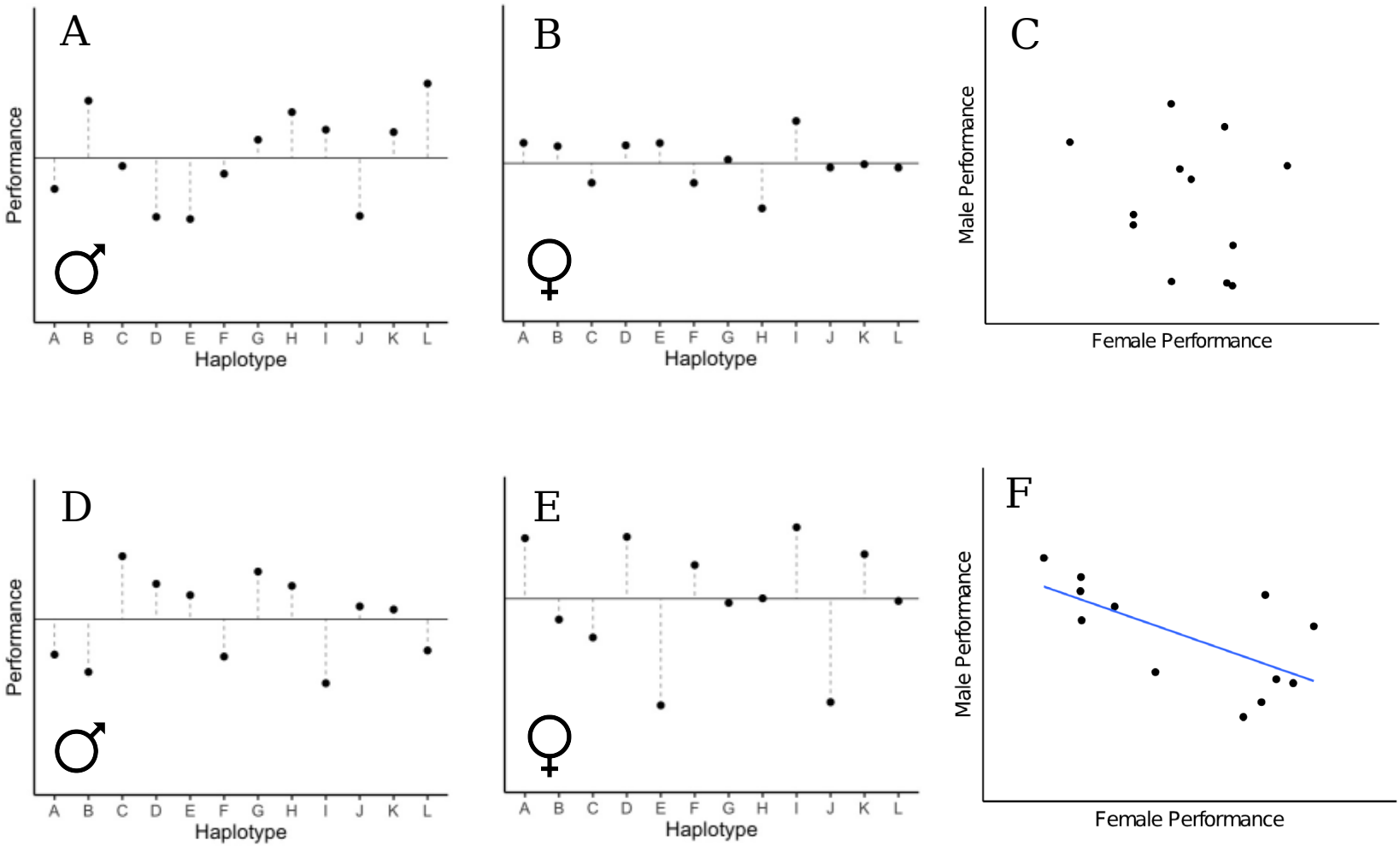
Figure 4. The effects of Mother's Curse may be masked if nuclear mutations are able to eliminate or compensate for the effects of male-specific mitochondrial mutations. However, expressing that mitochondrial genome alongside a novel nuclear genome will eliminate any such masking effects. While disrupting mito-nuclear compatibility may be predicted to cause decreased performance across both sexes, the decrease may be more severe in males (circles, solid line) than in females

(triangles, dashed line) if nuclear genes have been masking harmful male-specific mtDNA mutations. The hypothetical results depicted in the figure demonstrate how disrupting coevolved mito-nuclear genomes may reveal Mother's Curse effects that are not otherwise detectable.

Figure 5. The effects of mitochondrial genetic variation on performance can be subtle, and measuring a large number of different lines (A) is important to ensure that variation is not over- (B) or under-represented (C). Here, the mean performance across all haplotypes in this hypothetical data set is represented by the solid horizontal line, while each vertical dashed line illustrates deviation of that haplotype from the mean.







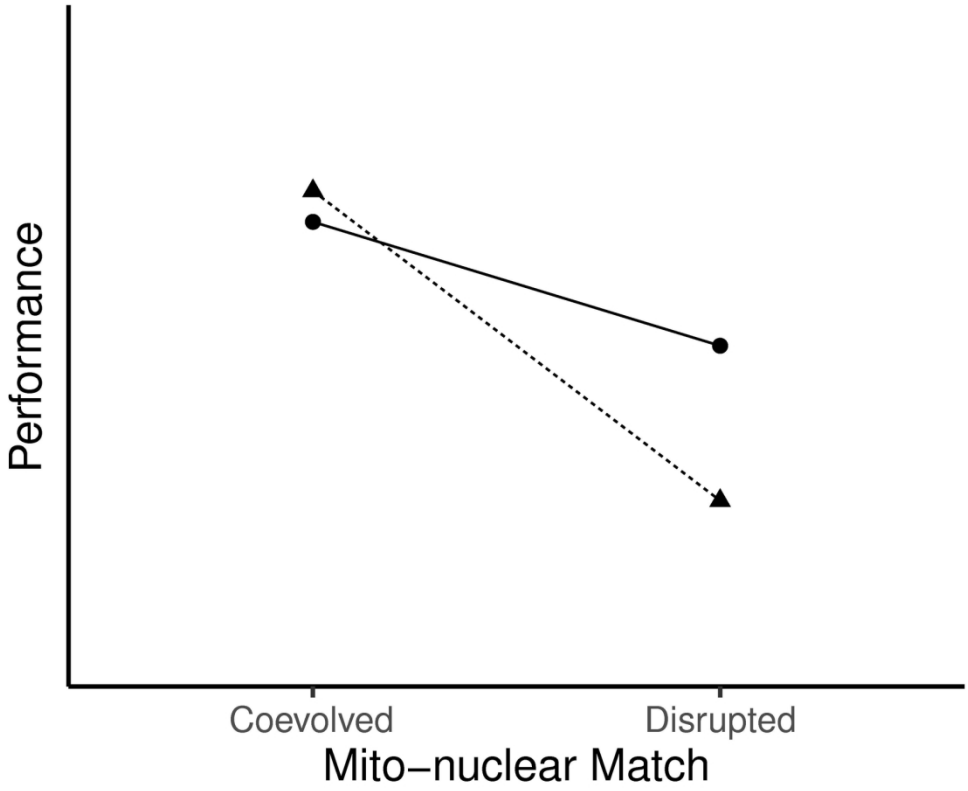


Figure 4. The effects of Mother's Curse may be masked if nuclear mutations are able to eliminate or compensate for the effects of male-specific mitochondrial mutations. However, expressing that mitochondrial genome alongside a novel nuclear genome will eliminate any such masking effects. While disrupting mito-nuclear compatibility may be predicted to cause decreased performance across both sexes, the decrease may be more severe in males (circles, solid line) than in females (triangles, dashed line) if nuclear genes have been masking harmful male-specific mtDNA mutations. The hypothetical results depicted in the figure demonstrate how disrupting coevolved mito-nuclear genomes may reveal Mother's Curse effects that are not otherwise detectable.

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