FROM THE COVER

Introducing mother’s curse: low male fertility associated with an imported mtDNA haplotype in a captive colony of brown hares

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Abstract
Mitochondrial DNA mutations create variation in the efficiency of the oxidative phosphorylation pathway and therefore cellular energy production. Mildly deleterious mutations may reduce the performance of sperm cells in particular, due to their high energy requirements and low number of mitochondria, yet have little or no effect on the viability of somatic cells or ova. Mutations will be maintained in the population, despite the fitness cost for males, because mtDNA is passed down the female line. We looked for this so-called mother’s curse effect in our captive colony of European brown hares. Significantly reduced male reproductive success was detected for a divergent haplotype that could be traced back to hares imported from a remote population. Median reproductive success for these hares was 0.17 compared to 0.49 for the indigenous haplotypes (Wilcoxon rank-sum, \(P = 0.002\)). No difference was detected for female reproductive success, nor were we able to find a nuclear DNA component to variation in male fertility. Our data are strong evidence for a mother’s curse effect persisting despite multiple crossings over seven generations. These data raise important issues relating to the reproductive fitness of small or intermixing populations and have particular implications for the management of populations for conservation.

Keywords: captive breeding, mother’s curse, mtDNA, reproductive success

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Introduction
Mitochondrial DNA has traditionally been used by population geneticists as the marker of choice to trace evolutionary patterns and historical processes (Avise et al. 1987; Zink & Barrowclough 2008). Many researchers have recently stressed the functional role of mtDNA, however, and called for a more direct application of mitochondrial data to issues in ecology and evolution (Ballard & Whitlock 2004; Gemmell et al. 2004; Dowling et al. 2008). All 13 protein coding genes of the vertebrate mitochondria comprise important components of the oxidative phosphorylation (OXPHOS) pathway. Changes in amino acid composition that affect the efficiency of OXPHOS will therefore have wide ranging implications for organismal fitness (da Fonseca et al. 2008). Whereas selection will quickly remove the majority of deleterious mutations, those causing only slightly deleterious changes in the efficiency of energy production may persist. Such mild changes are likely to affect male reproductive cells to a much greater extent than somatic or female reproductive cells due to their disproportionate energy requirements. Sperm cells have a high rate of energy expenditure for motility yet possess only a fraction of the mitochondria present in most other cells (Cummins 1998; Diez-Sanchez et al. 2003). Thus mtDNA mutations causing relatively minor decreases in OXPHOS efficiency can significantly reduce sperm function and therefore male fertility, but have no influence on the viability of ova due to their lower energy requirements. Female fertility is therefore unaffected. Owing to the maternal mode of inheritance for mtDNA, the mutant haplotype can be maintained in the population through the female line. This scenario has been termed the ‘mother’s curse effect’ (Gemmell et al. 2004).
Evidence for mother’s curse has been found in insects (Dowling et al. 2007a), chickens (Froman & Kirby 2005) and humans (Holyoake et al. 2001; Ruiz-Pesini et al. 2004). Despite these few examples, the question remains why this theoretically plausible phenomenon is not more commonly reported given its clear implications for the maintenance of variation in male fitness, the evolution of polyandry, and sperm competition theory. Gemmell et al. (2004) suggest the lack of detection may relate to many researchers overlooking it as a possibility to explain reduced population fitness, or that there may exist a number of compensatory mechanisms that mask such an mtDNA-based mother’s curse effect. Heteroplasmic of mtDNA within an individual may also play a role in buffering the effect (Chinnery et al. 2000). The deleterious mtDNA mutation must first reach a threshold frequency relative to other mtDNA variants within an individual and be present in the gametic tissue before reproductive success is compromised (Gemmell et al. 2004). Another potential mechanism involves the co-adaptation of nuclear encoded genes that are also involved in the OXPHOS pathway. Thus a mother’s curse effect may be masked by corresponding nucDNA compensatory mutations. In which case, intermixing of populations could disrupt such co-adapted gene complexes and reveal an mtDNA effect on male fertility (Dowling et al. 2008).

To test for a mother’s curse effect requires a long-term study population with detailed records of male breeding success that also includes experimental backcrosses with individuals from a divergent population. Our captive population of European brown hares fulfills these criteria. This captive colony was established in 1984 with hares of a central European origin (see Hartl et al. 1991). In an effort to reduce inbreeding effects, the colony was supplemented in 1998 with a number of individuals from northern Italy. Although it was unknown at the time, many of these individuals represented a divergent mitochondrial lineage that has since been identified as a distinct phylogeographic clade (Kasapidis et al. 2005; Stamatis et al. 2009). This unwitting experimental backcross provided us with the opportunity to test for a mother’s curse effect in a recently admixed population. We test whether the additional individuals imported from Italy possess divergent haplotypes to our resident colony derived from local Austrian populations. We then calculate the reproductive success for males of all haplotypes across multiple generations, therefore ensuring a randomly mixed nuclear background and the dilution of behavioural incompatibilities. Finally, we look for an association between mtDNA haplotype and male reproductive success. We expect a mother’s curse effect to be detected as a variation in male fertility associated with specific mtDNA haplotypes.

**Materials and methods**

**Captive colony and male breeding success**

Individual male hares came from the captive breeding colony at the Research Institute of Wildlife Ecology in Vienna, Austria. This breeding colony has existed since 1984 and comprises founding and supplementary individuals from the surrounding wild populations of European brown hares (Hartl et al. 1991) as well as descendents from eight hares (four males, four females) imported in 1998 from the Tuscan breeding colony in Italy. Over the breeding season, from early March to late October, individual males were paired for a period of 2 days with a single female which were otherwise kept in isolation. This was treated as a single breeding attempt. Males are mated to multiple females throughout the breeding season. If a pairing fails to result in a pregnancy, the female is subjected to further mating attempts, initially with the same male, and then with other individuals if failed reproduction persists. Individual male breeding success was calculated from detailed records of these mating attempts which were available for the breeding seasons of 1999 to 2005 (seven generations). These data files are a record of which individuals have been paired in the breeding program and allowed us to calculate the number of successful breeding attempts out of the total breeding attempts. For males in our analysis, we only included those with a minimum of five breeding attempts who had been paired with at least one female that had produced offspring. For female hares, we included only those that had undergone a minimum of five breeding attempts over their lifetime. We had enough data to calculate the reproductive success of 55 males based on between five and 36 breeding attempts (mean = 13.2 ± 0.95 SE) and 126 females with between five and 27 breeding attempts (mean = 9.89 ± 0.43 SE). These individuals were assigned to a haplotype based on either mtDNA sequence data (n = 28) or pedigree data of maternal lineages (n = 27).

**DNA extraction and PCR amplification**

Total genomic DNA was extracted using the GenElute™ Mammalian Genomic DNA Miniprep kit (Sigma-Aldrich) from muscle tissue of 28 of the males for which we calculated reproductive success. PCR amplification of a portion of the mitochondrial control region including the entire CR-1 was carried out according to Ben Slimen et al. (2007). Portions of two protein coding genes that have been implicated in male infertility (Ruiz-Pesini et al. 1998; Holyoake et al. 1999) were also amplified: a 453 bp segment of the mitochondrial...
ATPase sub-unit 6 from site 8142 to 8594; and a 409 bp segment of the mitochondrial NADH dehydrogenase sub-unit 2 from site 4066 to 4474 (Arnason et al. 2002). Reactions were carried out in 25 μL volumes and contained 1x PCR buffer (Qiagen), 200 μM each dNTP, 0.2 μM of each primer and 0.5 units of Taq polymerase. Samples were first denatured for 5 min at 94 °C followed by 35 cycles of 95 °C for 45 s, 53 °C for 45 s and 72 °C for 60 s and a final extension step of 72 °C for seven minutes. PCR products were gel purified using the ‘Wizard SV gel and PCR clean-up system’ (Promega) and sequenced by Macrogen Inc.

Data analysis

Sequences were edited and aligned by eye using the program BioEdit (Hall 1999). Sequences for all three loci were combined and a haplotype network was constructed using the program TCS (Clement et al. 2000). Individuals were assigned to haplotype groups based on sequence similarity. Associations between haplotype and male breeding success were assessed via a Wilcoxon rank-sum test. As a test of the potential for outbreeding depression at nuclear loci, the percentage breeding depression of fathers was compared to that of their sons that had also been included in the breeding program. This test ignores mtDNA and focuses purely on the outbreeding effect due to the nuclear component heritable from fathers to sons. The relationship was tested via least squares linear regression and male breeding success was also assessed via a Wilcoxon rank-sum test (one-tailed Z test, P = 0.033). The haplotype network (Fig. 1) showed four of the closely related haplotypes separated by just two to four mutational steps whereas the remaining haplotype (B) was 18 mutational steps from its nearest neighbour (haplotype A).

Due to the rarity of haplotypes C (n = 1), D (n = 1) and E (n = 3) and their close phylogenetic proximity to haplotype A (n = 33), all of these haplotypes were pooled into a single haplogroup (haplogroup A) and compared to individuals with the B haplotype (haplogroup B). Males belonging to haplogroup B (n = 17) and female breeding success was also assessed via a Wilcoxon rank-sum test. As a test of the potential for outbreeding depression at nuclear loci, the percentage breeding depression of fathers was compared to that of their sons that had also been included in the breeding program. This test ignores mtDNA and focuses purely on the outbreeding effect due to the nuclear component heritable from fathers to sons. The relationship was tested via least squares linear regression and male breeding success was also assessed via a Wilcoxon rank-sum test (one-tailed Z test, P = 0.033). The haplotype network (Fig. 1) showed four of the closely related haplotypes separated by just two to four mutational steps whereas the remaining haplotype (B) was 18 mutational steps from its nearest neighbour (haplotype A).

Results

Combined sequences from all individuals produced five distinct haplotypes from an alignment of 1204 basepairs displaying 21 variable sites. Genbank accession numbers for the ATP6 sequences are GU085213–GU085215 and for the ND2 sequences are GU085216–GU085219. Only two of the changes were nonsynonymous and both of these occurred in the ND2 gene. The first caused a change from a threonine to an alanine amino acid for haplotype B at residue 78 while the second altered an isoleucine to a valine for haplotype C at residue 105. The codon-based test of purifying selection, averaging over all sequence pairs, rejected the null hypothesis of strict-neutrality (dS = dN) in favour of the alternative hypothesis of purifying selection (dS < dN) (one-tailed Z test, P = 0.00). The haplotype network (Fig. 1) showed four of the closely related haplotypes separated by just two to four mutational steps whereas the remaining haplotype (B) was 18 mutational steps from its nearest neighbour (haplotype A).

Due to the rarity of haplotypes C (n = 1), D (n = 1) and E (n = 3) and their close phylogenetic proximity to haplotype A (n = 33), all of these haplotypes were pooled into a single haplogroup (haplogroup A) and compared to individuals with the B haplotype (haplogroup B). Males belonging to haplogroup B (n = 17)
had significantly lower reproductive success than the males of haplogroup A (median 0.17 vs. 0.49; Wilcoxon rank-sum test; \( z = -3.04; P = 0.002 \)). There was no difference in the median reproductive success of those individuals that were sequenced and those that had their haplotypes inferred from the pedigree (Wilcoxon rank-sum test; \( z = 0.90; P = 0.37 \)). The frequency distribution of reproductive success illustrates the high proportion of failed reproduction attempts for males of haplogroup B relative to A (Fig. 2). The low male reproductive success for haplogroup B persisted across multiple generations of mixing with the resident hares (Table 1). Whereas reproductive success for haplogroup A males remained constant across time, the success rate for haplogroup B tended to decline after the F1 crosses, although not significantly (\( F = 0.63, P = 0.54 \)). Conversely, there was no association between female reproductive success and haplogroup (Wilcoxon rank-sum test; \( z = -1.35; P = 0.18 \)). There was also no effect for average litter size per successful mating between each haplogroup (Wilcoxon rank-sum test; \( z = 1.4483, P = 0.1475 \)). A nuclear DNA component to suppressed male reproductive success would be directly heritable from fathers to sons yet we found no relationship between the fathers’ breeding success and that of their sons (Fig. 3) (\( R^2 = 0.0075, F = 1.29; P = 0.26 \)).

The phylogenetic tree (Fig. 4) shows a clear separation of the northern Italy clade from the central European clade as described by Kasapidis et al. (2005). All haplotypes from our haplogroup A category correspond to sequences in the sub clade B-VII whereas our haplotype B is identical to the Leu2 sequence from clade B-III. There is strong bootstrap support for the separation of these two clades. Clade B-III has been previously identified as of northern Italian origin (Pierpaoli et al. 1999; Kasapidis et al. 2005) which agrees with the known source population of the imported hares.

### Table 1

<table>
<thead>
<tr>
<th></th>
<th>Pure bred males</th>
<th>F1 males</th>
<th>F2+ males</th>
</tr>
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<tbody>
<tr>
<td>Haplogroup A (Central Europe)</td>
<td>0.42 ± 0.04 (( N = 343 ))</td>
<td>0.40 ± 0.16 (( N = 54 ))</td>
<td>0.55 ± 0.07 (( N = 88 ))</td>
</tr>
<tr>
<td>Haplogroup B (Northern Italy)</td>
<td>0.24 ± 0.05 (( N = 172 ))</td>
<td>0.25 ± 0.21 (( N = 29 ))</td>
<td>0.16 ± 0.13 (( N = 37 ))</td>
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\( N \) = number of mating attempts in each category. F2+, Hybrid generations following the first cross.

### Discussion

We found a clear association between a particular mtDNA haplotype and reduced male reproductive success in a captive colony of European brown hares. We believe this is one of the few non-human examples of the mother’s curse effect, in which a maternally inherited gene adversely affects the reproductive success of sons. Only 47\% of the males from haplogroup B produced offspring, compared to 90\% of males from haplogroup A (Fig. 2). A simple outbreeding explanation for our data seems unlikely for two main reasons. First, the effect persisted despite 7 years of backcrossing and
mixing of nucDNA. A role for mating behaviour incompatibility would then only hold if such a complex phenotypic trait was encoded entirely by mtDNA. Genes on the Y-chromosome may influence breeding success and largely avoid recombination, yet their paternal mode of inheritance means they are unlikely to be responsible for the observed mtDNA-linked effect. Second, an outbreeding explanation should involve a strong nucDNA component (Lynch 1991), yet we found no such association between the reproductive success of fathers and sons (Fig. 3). Moreover, females of haplogroup B showed no difference in reproductive success compared to other females. This directly links the divergent mtDNA with reduced male reproductive success. A mother’s curse effect therefore seems highly plausible. Our results from a well studied captive colony raise important issues relating to the reproductive fitness of small or intermixing populations and have particular implications for the management and conservation of threatened populations.
Our findings fit expectations for what should be observed given the mother’s curse hypothesis (Gemmell et al. 2004). Slightly deleterious mutations in mtDNA OXPHOS genes can strongly affect the performance of sperm cells due to their high energy requirements for motility. Individual males harbouring these mutations will have poorly performing sperm and therefore reduced reproductive success relative to others, yet the mutations will be retained in the population due to the maternal inheritance of mtDNA. The nonsynonymous mutations will be retained in the population due to the reduced reproductive success relative to others, yet the mutations will have poorly performing sperm and therefore sperm cells due to their high energy requirements for OXPHOS genes can strongly affect the performance of some male hares, but a reduction in sperm motility caused by minor disruption to OXPHOS is a strong candidate. Interestingly, there was no effect for average litter size between each haplogroup. This suggests that the energetic consequences of the mtDNA differences reduce the chance of fertilization but not the number of zygotes formed in successful matings. The obvious next step is to directly link the B haplotype with reduced sperm function via tests of metabolic output and motility (Ruiz-Pesini et al. 1998; Froman et al. 1999).

We know of only a handful of studies that report a mother’s curse effect (Moore & Reijo-Pera 2000; Ruiz-Pesini et al. 2000; Holyoake et al. 2001; Froman & Kirby 2005; Dowling et al. 2007b). In wild populations, it is difficult to quantify the success of mating attempts and to obtain genetic samples from all males that could potentially be involved in reproduction. These problems are resolved in captive breeding programs, as detailed information is usually recorded in stud books and pedigrees. However, mtDNA has traditionally been used purely as a marker in wildlife population studies, and only recently has attention turned to the functional effects of mtDNA mutations on individual and population fitness (Moore & Reijo-Pera 2000; Ruiz-Pesini et al. 2000; Carra et al. 2004; Liau et al. 2007). There is therefore considerable scope to look for similar mtDNA effects on breeding success in other captive animal populations. Another reason for the paucity of examples and some studies even reporting no relationship in natural populations (Pereira et al. 2007; Friberg & Dowling 2008) is the possible masking effect created by the interplay between mtDNA and nuclear genes (Dowling et al. 2008). Again, these effects might be revealed in captive breeding programs, which could inadvertently disrupt such compensatory intrapopulation adaptations.

The implications of our findings for conservation are important. We have demonstrated the persistence of a mitochondrial haplotype that detrimentally affects male reproductive success in a small population of mammals. This increased variation in paternity, which persists largely independent of selection (but see Wade & Brandvain 2009), further reduces the effective size of small populations beyond that expected based on the census size. A traditional goal for the genetic management of endangered populations is to reduce inbreeding effects by increasing genetic diversity via translocations, reintroductions and captive breeding from multiple source populations. There are many examples of how such genetic restoration has increased population fitness (see reviews in Edmands 2007; Tallmon et al. 2004); however, studies such as ours provide a cautionary tale. The benefits of population admixture may be compromised by the introduction of a mother’s curse haplotype or by the breakdown of co-adapted mito-nuclear genomes (Willett & Burton 2001; Burton et al. 2006; Ellison & Burton 2008). Incorporating mtDNA effects into models of population viability will clarify how much the goal of maximising genetic diversity in these populations should be tempered by such concerns (Allendorf & Ryman 2002). Future studies of captive and natural populations are needed to establish the prevalence of the mother’s curse effect, including its possible role in explaining cases of skewed paternity and poor recovery of threatened populations.

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References


Lagomorpha) with different coat colours. *Mammalian Biology, 72*, 224–239.


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The authors’ individual research interests include: application of molecular genetic techniques to issues of conservation (S.S.); physiological mechanisms underlying animal behaviour and ecology (C.T.); and population and evolutionary genetics of animals (F.S.). In common, all three authors have recently become interested in the functional effects of mtDNA variation on reproduction and fitness.