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Mitochondrial haplotypes are not associated with mice selectively bred for high voluntary wheel running

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ABSTRACT

Mitochondrial haplotypes have been associated with human and rodent phenotypes, including nonshivering thermogenesis capacity, learning capability, and disease risk. Although the mammalian mitochondrial D-loop is highly polymorphic, D-loops in laboratory mice are identical, and variation occurs elsewhere mainly between nucleotides 9820 and 9830. Part of this region codes for the $tRNA^{Arg}$ gene and is associated with mitochondrial densities and number of mtDNA copies. We hypothesized that the capacity for high levels of voluntary wheel-running behavior would be associated with mitochondrial haplotype. Here, we analyzed the mtDNA polymorphic region in mice from each of four replicate lines selectively bred for 54 generations for high voluntary wheel running (HR) and from four control lines (Control) randomly bred for 54 generations. Sequencing the polymorphic region revealed a variable number of adenine repeats. Single nucleotide polymorphisms (SNPs) varied from 2 to 3 adenine insertions, resulting in three haplotypes. We found significant genetic differentiations between the HR and Control groups ($F_{st} = 0.779$, $p \leq 0.0001$), as well as among the replicate lines of mice within groups ($F_{sc} = 0.757$, $p \leq 0.0001$). Haplotypes, however, were not strongly associated with voluntary wheel running (revolutions run per day), nor with either body mass or litter size. This system provides a useful experimental model to dissect the physiological processes linking mitochondrial, genomic SNPs, epigenetics, or nuclear-mitochondrial cross-talk to exercise activity.

1. Introduction

Mitochondria are fundamental for cellular energy production via oxidative phosphorylation through the four respiratory enzyme complexes that form the electron transport chain (Saraste, 1999). Although the nuclear DNA (nDNA) and mitochondrial DNA (mtDNA) both encode the respiratory complexes, the mtDNA genome specifically codes for the12S and 16S rRNAs, the 22 tRNAs required for mitochondrial protein synthesis, and the 13 polypeptides involved in oxidative phosphorylation. The function of the 13 polypeptides includes regulating mitochondrial transcription and expression of the genome (i.e., the control region or D-loop; Sbisa et al., 1997). The structure of the mitochondrial D-loop is highly polymorphic among mammals and therefore commonly analyzed for variations (Bibb et al., 1981; Brown et al., 1986; Saccone et al., 1987; Sbisa et al., 1997; Pesole et al., 1999). Interestingly, D-loops in strains of laboratory house mice are identical (Bayona-Bafaluy et al., 2003) and variation occurs elsewhere, that is outside of the D-loop between nucleotides 9820 and 9830 of the mtDNA genome. Part of this particular region codes for the $tRNA^{Arg}$ gene and is not highly conserved among species (Johnson et al., 2001). This region has been associated with differing amounts of reactive oxygen species (ROS) production by mitochondrial haplotype variants (Moreno-Loshuertos et al., 2006).

Mitochondrial variants likely have significant consequences for fitness and metabolic processes because mitochondria are responsible for cellular energy production (William et al., 1995; Ballard and Whitlock, 2004). Indeed, mitochondrial haplotypes have been associated with various fitness-related human and rodent phenotypes, such as sperm motility in mice (Ruiz-Pesini et al., 2000; Montiel-Sosa et al., 2006; Nakada et al., 2006), age-related hearing loss in mice (Johnson et al.,

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2001), and changes in cognition with aging in mice (Roubertoux et al., 2003), as well as longevity in humans (Tanaka et al., 1998; Coskun et al., 2003; Niemi et al., 2003), and disease risks in both mice and humans (Wallace, 2005). Mitochondrial haplotypes have also been associated with various metabolic-related phenotypes, such as non-shivering thermogenesis capacity in the greater white-toothed shrew (Fontanillas et al., 2005), climatic adaptation in humans (Ruiz-Pesini et al., 2004; Wallace, 2005), ROS production in cell lines from mice (Moreno-Loshuertos et al., 2006), and metabolic diseases in mice and humans (Wallace, 2005).

The metabolic consequences of mitochondrial DNA variants occur because they influence mitochondrial densities and number of mtDNA copies in mice (Moreno-Loshuertos et al., 2006). Consequently, such variants confer differences in aerobic exercise capacity (Calo and Vona, 2008). In addition, muscle mitochondria directly respond to physical activity and training (Marcuello et al., 2005; Eluamai and Brooks, 2013; Barbieri et al., 2015). To test for mitochondrial haplotype responses to activity and training, we analyzed the polymorphic region of the mtDNA genome in mice from four replicate lines that have been selectively bred for 54 generations for high voluntary wheel running and four non-selected control lines (Swallow et al., 1998; Garland et al., 2011a, 2011b). These selected lines of mice belong to an ongoing artificial selection experiment and were derived from an outbred Hsd:ICR strain (Rice and O'Brien, 1980; Swallow et al., 1998; Carter et al., 1999). The mtDNA genome of the Hsd:ICR strain is not available from GenBank of the National Center for Biotechnology Information (NCBI; http://www.ncbi.nlm.nih.gov/), but mtDNA genomes for two sister inbred strains (i.e., SWR/J and SJL/J) and one outbred strain (i.e., NIH) related to the outbred ICR are available (Rice and O'Brien, 1980). Alignment of these mtDNA genomes indicated that they were essentially identical, with the exception of a single nucleotide polymorphism (SNP) between nucleotides 9820 and 9830. The SNPs within this region contain 9 or 10 adenine repeats. The number of adenine repeats between nucleotides 9820 and 9830 is highly polymorphic in laboratory mice, varying between 8, 9, and 10 (Johnson et al., 2001). A single base pair change in this region has been shown to be functionally important, based on studies of aminoacylation in yeast (Liu et al., 1999) and hearing impairment in mice (Johnson et al., 2001). These published results provide evidence of mtDNA variation in ICR mice, which provides phenotypic variation on which artificial selection can act. Thus, we hypothesized that the capacity for high levels of voluntary, aerobically supported wheel running would be associated with mitochondrial haplotypes in laboratory house mice.

2. Materials and methods

2.1. Study animals

The founding population of mice in the artificial selection experiment was 112 males and 112 females from the outbred Hsd:ICR strain (Rice and O'Brien, 1980; Swallow et al., 1998; Carter et al., 1999). Descriptions of the High Runner (HR) lines of mice can be found in previous publications (Rhodes et al., 2005; Garland et al., 2011a, 2011b; Wallace and Garland Jr, 2016). Briefly, in the selected lines, the male and female from each family that ran the most number of revolutions run on days 5 and 6 of a 6-day period during where they had access to running wheels (Wahman-type activity wheels, 1.12 m circumference) at ~7–9 weeks of age were chosen as breeders for the next generation. In the control lines, a random male and female from each family were chosen as breeders for the next generation. Within all lines, male and female pairings were random with the restriction that siblings were not mated to each other. Ten pairs of mice (families) were used to propagate the next generation of each line in each generation as described more fully in Swallow et al. (1998). For this study, tail tips were collected in adult females from generation 54 after voluntary wheel running metrics were measured (see Mitochondrial DNA haplotyping below). Institutional Animal Care and Use Committee of the University of California Riverside, which is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care (AUP A-20080018), approved all animal care and handling procedures.

2.2. Phenotypes

We analyzed average values for wheel-running traits on days 5 and 6 of wheel access, as used to choose breeders in the routine selection protocol (above). We also collected and analyzed body mass and litter size (see Statistical analysis below).

2.3. Mitochondrial DNA haplotyping

mtDNA was extracted from 3 mm tail tips using Proteinase K (Viagen Biotech, Inc. Los Angeles, CA) and Direct PCR (Viagen Biotech, Inc. Los Angeles, CA) from 9 to 11 mice of each line of mice. We designed primers to amplify a PCR amplicon to cover the polymorphic region, which includes the $tRNA^{Arg}$ gene. The primers were (*1*) forward: 5′ - GAA GCC GCA GCA TGA TAC TGA CA – 3′, and (*2*) reverse: 5′- AGG TTG AAG AAG GTA GAT GGC ATA – 3′, which resulted in PCR products with an average size of approximately 500 bp. PCR reactions contained 20 ng DNA, 1 µl of each primer (5 µM concentration), 10 mM Tris-HCl, pH 8.3, 200 µM dNTPs and 0.2 µl titanium Taq DNA polymerase (PE Applied Biosystems) in a total volume of 25 µl. The DNA was initially denatured at 94 °C for 1 min, followed by 32-step cycles of denaturing at 93 °C for 40 s, annealing at 68 °C for 50 s, extension at 68 °C for 30 s and a final extension at 68 °C for 7 min in the GeneAmp PCR System 9700 (PE Applied Biosystems). PCR products were cleaned using Qiagen MinElute filter plate on the Qiagen BioRobot 3000. Cleaned amplicons were sequenced using the ABI 3730 DNA analyzer.

Sequenced mtDNA amplicons (i.e., FASTA files) were analyzed by base calling and fragment assembly using the Phred and Phrap, software package, respectively (Ewing and Green, 1998). All trace files were based called using Q score ≥ 20 and assembled by overlapping a minimum continuous stretch of 50 bp to ensure the quality of the consensus. In addition, we performed manual curation based on the trace by Consed to identify single nucleotide substitutions in individuals to confirm the existence of high-quality sequence reads in the region before calling the nucleotide substitution or other sequence alteration as a variant (Gordon et al., 1998). Assembled sequences were aligned and visualized by T-coffee with Jalview (Notredame et al., 2000; Waterhouse et al., 2009). The consensus sequence for each individual is derived from the samples sequenced in this study.

2.4. Statistical analysis

Mitochondrial haplotypes were compared between the HR group and the Control group by hierarchical analysis of molecular variance (AMOVA) using Arlequin ver 3.5.2.2 (Excoffier and Lischer, 2010). Hierarchical AMOVA estimates the genetic differentiation (i.e., fixation index) among groups (F_{ST}), among populations (i.e., replicate lines of mice) within groups (F_{SC}), and within populations (F_{CT}). These fixation indices have a theoretical minimum of 0 (indicating no genetic divergence) and a theoretical maximum of 1 (indicating fixation for alternative alleles, or SNPs, in different groups or populations; Hartl and Clark, 2007). Indices can be qualitatively interpreted as 0–0.5 little genetic differentiation, 0.05–0.15 moderate genetic differentiation, 0.15–0.25 great genetic differentiation, and > 0.25 very great genetic differentiation (Wright, 1984). Significance associated with the fixation indices were evaluated through random allelic/SNP permutation procedures (10,000 permutations) in Arlequin (Excoffier and Lischer, 2010).

Wheel-running activity (i.e., mean number of revolutions run per day, mean minutes run per day, and mean maximal speed recorded for any 1-minute interval), litter size, and body mass were compared

Table 1
Number of haplotypes detected and their associated single nucleotide polymorphism (SNP) from the highly polymorphic region of the mtDNA encoded *tRNA^{Arg}* gene region in the 8 lines of mice sampled.

Haplotype	Number of adenine repeats
1	9
2	10
3	11

among the three haplotypes by restricted maximum likelihood (REML) generalized least squares fit using SAS procedure MIXED (SAS Institute, Cary, NC, USA). Main effects were mtDNA haplotype (3 categories), line (N = 8), wheel freeness, and the line x mtDNA interaction (wheel freeness was transformed by raising to the 0.7 power to make it more normal). Initial analysis indicated that the interaction term, mtDNA x line was not significant, so it was not included in the final statistical models. Separate models were generated for wheel-running activity, litter size, and body mass.

3. Results

Sequencing of the polymorphic region revealed a variable number of adenine repeat insertions (i.e., SNP) in the *tRNA^{Arg}* gene of mice sampled. SNPs varied from two to three adenine repeat insertions, resulting in 3 haplotypes (Table 1; Fig. 1). Hierarchical AMOVA indicated that haplotypes varied significantly among mice bred for high running compared to controls (i.e., linetype), and among populations (i.e., each of the replicate lines) within groups, but not within populations (Table 2).

Mitochondrial haplotype was not associated significantly with the mean number of revolutions run per day ($F_{2,70} = 2.39$, $p = 0.10$), mean minutes run per day that showed any wheel revolutions, which is a measure of the amount of time spent running per day ($F_{2,70} = 2.59$, $p = 0.08$; Fig. 2), mean running speed (revolutions/active minutes) ($F_{2,70} = 0.72$, $p = 0.49$), or mean maximal speed recorded for any 1-minute interval ($F_{2,70} = 2.29$, $p = 0.11$). Mitochondrial haplotype also was not associated with body mass at weaning ($F_{2,70} = 0.55$, $p = 0.58$), body mass at the start of wheel access ($F_{2,70} = 0.90$, $p = 0.41$), body mass at end of day 6 of wheel access ($F_{2,70} = 0.66$, $p = 0.52$), or their litter size at weaning ($F_{2,69} = 0.42$, $p = 0.66$).

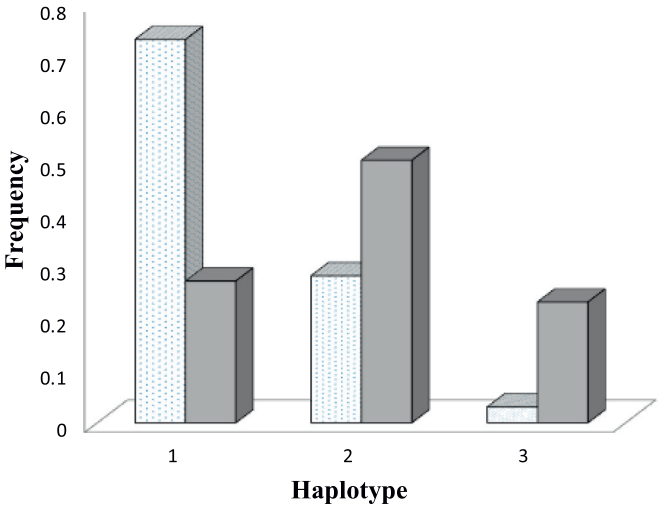

Fig. 1. Frequency distributions of the mitochondrial haplotypes detected between 4 replicate Control (pooled data, stippled) lines and 4 replicate Selected lines (pooled data, gray) in laboratory mice from a long-term breeding experiment (Swallow et al., 1998).

Table 2
Hierarchical analysis of molecular variance (AMOVA) in the highly polymorphic region of the mtDNA encoded *tRNA^{Arg}* gene region in two groups of mice (i.e., Control and Selected).

Source of variation	*d.f.*	Sum of squares	Percentage of variation	Fixation Index	*P*
Among groups (i.e., linetype)	1	4.682	8.76	$F_{ST} = 0.779$	< 0.0001
Among populations (i.e., lines) within groups	6	18.779	69.11	$F_{SC} = 0.757$	< 0.0001
Within populations	76	7.051	22.13	$F_{CT} = 0.088$	0.336

4. Discussion

We investigated whether mitochondrial genetic background influences the performance metrics of mice selectively bred for high voluntary wheel running activity. Our key finding was that mitochondrial haplotypes are significantly differentiated between the four HR and four Control lines, presumably due to the past selection history. However, haplotypes were not strongly associated with wheel-running behavior in an analysis controlling for variation in mean levels among the eight lines. To our knowledge, this is the first investigation of whether mitochondrial haplotypes are associated with a behavior.

Fifty-four generations of directional selection or non-selective breeding resulted in fixation of mitochondrial haplotypes in two of four HR lines of mice and in all four Control lines. Most of the Control lines have haplotype 1 (9 adenine repeats), except Control Line 5, which is fixed with ten adenine repeats (haplotype 3). A somewhat different fixation of mitochondrial haplotype pattern emerged in the HR mice, as two HR lines (6 and 7) expressed haplotype 2, one HR line (8) expressed haplotype 3, and one HR line (3) expressed haplotype 1 (Table 3). Overall, this pattern suggests that haplotypes 2 and 3 are associated with higher levels of wheel running (Fig. 2). If so, then the reason for lines 5 (Control) and 3 (HR) to vary from their respective linetypes is unclear. It is possible that genetic drift obscured any effects of selection, or that alternative mechanisms have been employed to achieve elevated wheel running (Garland et al., 2011a). With respect to the latter, it is important to note that line 3 (HR) is fixed for a mutation that causes a 50% reduction in hindlimb muscle mass while doubling mass-specific muscle aerobic capacity, among other effects (Garland Jr et al., 2002; Kelly et al., 2013), providing support for the concept that different evolutionary solutions may emerge within distinct mitochondrial haplotypes.

Given that laboratory mice show polymorphism in adenine repeats within this gene (Johnson et al., 2001), the fact that three of the four Control lines are fixed for a particular haplotype likely stems from founder effects or subsequent genetic drift. Small effective population sizes are highly susceptible to genetic drift (Kimura and Ohta, 1969; Whitlock, 2000; Kliman et al., 2008), and drift also appears to have played an important role in the evolutionary trajectory of the mini-muscle phenotype mentioned above (Garland et al., 2002).

The current study findings partially support the hypothesis that the capacity for high levels of voluntary wheel-running activity is associated with certain mitochondrial haplotypes. These results are somewhat consistent with the findings that mitochondrial haplotypes seem to be associated with exercise, especially endurance types. Specifically, some studies reported that certain mitochondrial haplotypes are associated with elite human endurance athletes (Castro et al., 2007; Nogales-Gadea et al., 2011; Kim et al., 2011; Mikami et al., 2013; Maruszak et al., 2014), but this association is controversial (Eynon et al., 2011).

The current study suggests that those mice with haplotype 2 or 3 mitochondria might also produce greater amounts of ROS. Recall that most of the lines of HR mice have haplotype 2 or 3. Rezende et al.

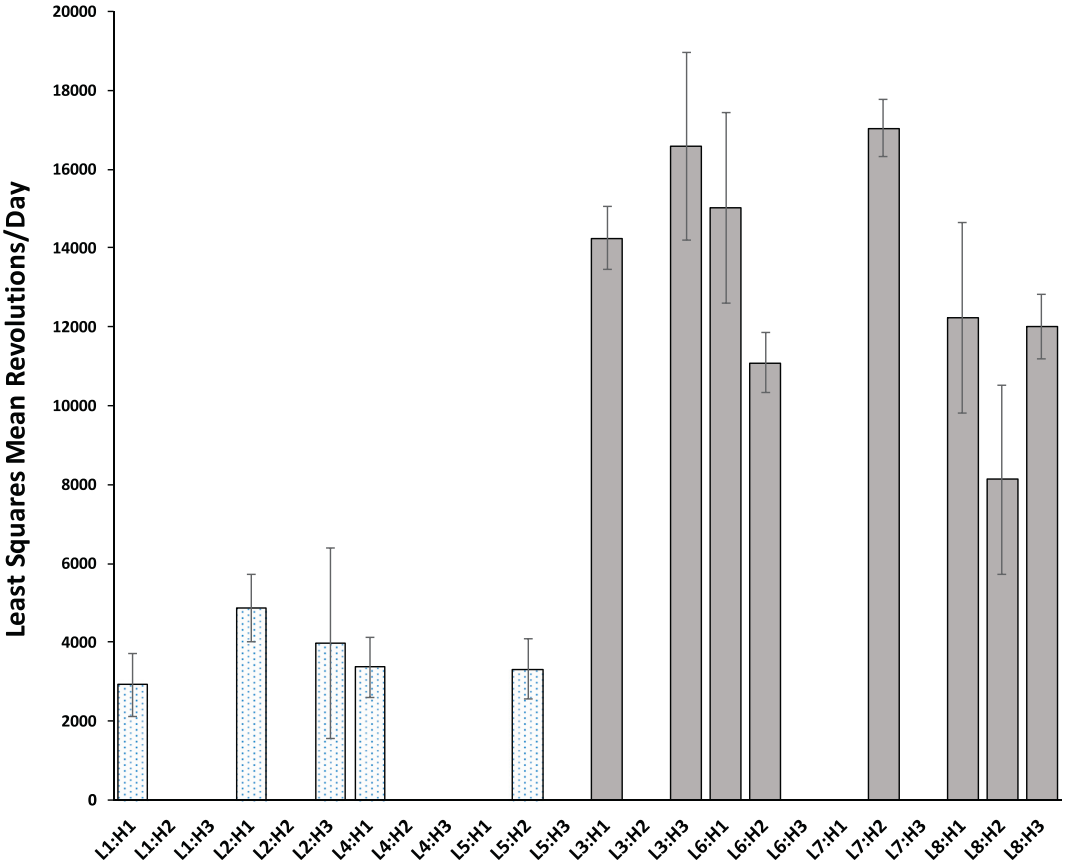

Fig. 2. Least squares mean revolutions/day, scored as mean values for days 5 & 6 of a 6-day period of wheel access (Swallow et al., 1998) in lines of mice separated by mtDNA haplotype (L:H). Wheel circumference = 1.12 m. Stippled bars - Control lines, Gray bars - Selected lines. Error bars represent standard error of the means.

Table 3
Haplotypes detected in the 8 lines of mice sampled. HR – high runner.

Genetic group (i.e., Linetype)	Population (i.e., Line)	n	Haplotype		
			1	2	3
Control	1	9	9		
	2	9	8		1
	4	11	11		
	5	11		11	
HR	3	10	9		1
	6	11	1	10	
	7	11		11	
	8	11	1	1	9

(2005) previously showed that the HR lines of mice have higher running speeds and higher maximal metabolic rate (MMR) during voluntary exercise compared with the Control lines. It is generally accepted that increased metabolic rate leads to increased ROS production, especially in muscle (Bejma and Ji, 1999; Ji, 1999). That is, individuals might have increased oxidative damage resulting from carrying haplotype 2 and 3 mitochondria (Moreno-Loshuertos et al., 2006), as well as from increased exercise activity (i.e., metabolic rates are higher with increased exercise activity; Gleeson et al., 1982; Lennon et al., 1985; Rezende et al., 2005), resulting in a costly pleiotropic association between high voluntary wheel running and mitochondrial haplotype. Previous studies on antioxidant defenses in these lines of mice showed (1) a negative correlated response of superoxide dismutase-2 (SOD-2) activity (Thomson et al., 2002), (2) but not elevated antioxidant enzyme activity (Vaanholt et al., 2008), and (3) control female mice housed with access to wheels had higher mRNA levels for two antioxidant enzymes, catalase (CAT) and SOD-2 when compared to HR females housed with access to wheels (Bronikowski et al., 2002). Further investigations are needed to clarify the link between ROS and haplotypes, and these lines of mice may prove particularly useful in that regard.

5. Conclusion

In conclusion, we analyzed the polymorphic region of the mtDNA genome in mice that have been selectively bred for 54 generations for high voluntary wheel running and their non-selected control lines. Our results suggest that certain haplotypes might be associated with voluntary wheel running activity. Mitochondrial haplotypes might be associated with other physiological traits, such as MMR, given that the mtDNA region sequenced influences respiratory performance (i.e., increased mitochondria densities) (Marcuello et al., 2009). This system might provide an experimental model to dissect the physiological traits linking mitochondrial (Manev and Dzitoyeva, 2013; Castegna et al., 2015), genomic SNPs (Bouchard et al., 2011; Kelly and Pomp, 2013; Xu and Garland, 2017), epigenetics (Barrès et al., 2012; Manev and Dzitoyeva, 2013; Brown, 2015; Grazioli et al., 2017), or nuclear-mitochondrial cross-talk (Safdar et al., 2011; Castegna et al., 2015) to levels of physical activity (Lightfoot et al., 2018).

Author contributions

BWMW and TG conceived the study. BWMW extracted DNA, designed primers, and PCR amplified the target mtDNA sequence. WCY assembled the sequenced PCR amplicons. HS and THM collected voluntary-wheel running and phenotypic data. BWMW analyzed the results and drafted the manuscript. All authors reviewed, edited and approved the final manuscript.

Conflict of interests

All authors declare no conflict of interest.

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