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The effect of mitochondrial DNA on behaviours and life history traits in Seed beetles (*Callosobruchus maculatus*).

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ABSTRACT

Recent studies have documented both interspecific and intraspecific variation in mitochondrial DNA (mtDNA). Due to only maternal inheritance and lack of recombination, this variation has been considered neutral and mitochondrial genes have therefore thought to be unselected for. This theory is currently being revised and replaced with adaptive evolution as the cause of this variation. The mitochondria has since long been known as the "cellular power plant" as it through oxidative phosphorylation (OXPHOS) generates most of the cells ATP in all animals. It has earlier been shown that there is a relationship between mtDNA and energy expenditure, however any link to behaviour and life history traits has remained unverified. By using nine introgression lines of Seed beetles (*Callosobruchus maculatus*) I have experimentally shown that activity is partly under influence of the mtDNA. In addition, I retained data supporting the theory of life span and adult weight at emergence to be under influence of variable nuclear and mitochondrial interactions. Taken together, my results highlight the relative importance of mtDNA variation for variation in behaviours and life history traits. In addition, these results further validate the non-neutral theory of mtDNA, and the importance mitochondrial DNA might have on the evolution of variation in consistent behaviours (i.e personality).

Keywords**:** mtDNA, mitochondria, *Callosobruchus maculatus,* mito-nuclear interaction, behaviours, life history traits.

INTRODUCTION

The mitochondrion is well-known as the "cellular power plant" where it through oxidative phosphorylation (OXPHOS) generates most of the cellular ATP in animals. The respiratory complexes active in the OXPHOS contain nucleus-encoded subunits; however, mitochondrial DNA (mtDNA) plays an important part for the mitochondria and mitochondrial build up (Schon *et al.* 1997). In addition to providing ATP for metabolism the mitochondrion has been shown to be involved in additional processes, such as signaling and cellular differentiation (Siskind *et al.* 2002, Garcia-Ruiz *et al.* 2002, van Blitterswijk *et al.* 2003), as well as control of the cell cycle and cell growth (Hardie 2005).

Mitochondrial DNA (mtDNA) has traditionally been thought to be selectively neutral due to the strict maternal transmission and the lack of recombination. However, this hypothesis has recently been revised and also mtDNA (and not only nuclear DNA) has been shown to be under selection (Ballard and Whitlock 2004, Dowling *et al.* 2008, Arnqvist *et al.* 2010, Innocenti *et al.* 2011). The view of sequence variation in the mtDNA being an accumulation of neutral mutations has in recent times been challenged by the idea that non-neutral polymorphisms is maintained in the genome by recurrent adaptive evolution (Bazin *et al.* 2006, Maklakov *et al.* 2006, Dowling *et al.* 2008, Stewart *et al.* 2008, Arnqvist *et al.* 2010). In addition to adaptive evolution, selection on mito-nuclear interactions is becoming a more investigated possible route for maintaining non-neutral mtDNA polymorphism (Rand *et al.* 2004, Arnqvist *et al.* 2010, Dowling *et al.* 2010).

Mitochondrial DNA show both interspecific and intraspecific variation in manners that challenge the traditional view (i.e. that mtDNA is unselected). For example Bazin *et al*. (2006) draw the conclusion of mitochondrial genes being selected for from a large scale comparative genomic approach including ~3000 animal species. Further, there is accumulating evidence that such selection occurs; this includes studies ranging from enzyme function (Ballard *et al.* 2007) to phenotypic variation in life history traits or fitness to naturally occurring and distinct mtDNA haplotypes (Dowling *et al.* 2007a).

With increased research on the cause of mtDNA variation and the accumulated knowledge of it being under selection, questions regarding the ecological or functional meaning this variation might impose on species and individuals are raised. For example, variation in mtDNA by itself, or in interaction with the nuclear genome, has been shown to cause differences in cellular metabolism (Wallace 1994, Ballard *et al.* 2007, Rocher *et al.* 2007, Smith *et al.* 2010, Arnqvist *et al.* 2010). Such variation is likely to impose variation in behaviors linked to these genes.

Different behaviours, for example activity and exploration, have been described for a wide range of species; different behaviours have also been observed to vary consistently within populations (Gosling 2001, Wolf *et al.* 2007). Further it has been shown that there is a genetic basis for heritability of behaviours (Van Oers *et al.* 2004a, Van Oers *et al.* 2004b, Fidler *et al.* 2007).

Despite the potential for influence on behaviours such as activity, very little is known about how mtDNA variation affects variation in behaviours. The potentiality of life history traits being affected by mitochondrial polymorphisms has been subject to investigation (reviewed in Ballard and Whitlock 2004), but still little is known. Therefore, I in this study aim to see if such a link exists by using Seed beetles (*Callosobruchus maculatus*), a well studied species within evolutionary ecology (Messina 1993, Fox *et al.* 2004, Edwardsson & Tregenza 2005, Dowling *et al.* 2007b, Bilde *et al.* 2009, Dowling *et al.* 2010, Arnqvist *et al.* 2010). I experimentally intend to investigate whether different mtDNA haplotypes, by themselves or in interaction with the nuclear genome, responds to differences in activity, life span and adult weight at emergence. Life span has been shown to be affected by the mitochondrial and nuclear interaction in *Drosophila melanogaster* (Maklakov *et al.* 2006). Adult weight at emergence, with its likely link to growth rate, is anticipated to vary as an effect of variation in mtDNA. I consider variations in mtDNA and thereby variations in metabolism to be of importance for activity and life history traits.

If mtDNA does have a substantial effect on behavior and life history traits it would indicate that the ability for these traits to evolve is lower as opposed to non-mitochondrial related traits. The reasoning behind this would be a non-additive genetic variance by either epistasis (mito-nuclear interaction) or dominance (mtDNA). Investigating such links may also further our understanding of the evolution of variation in consistent behaviours (i.e personalities) and the role mitochondrial DNA might have.

MATERIAL & METHODS

Study species

Seed beetles are widely distributed pests on stored legumes, whose larvae develop inside beans (Edvardsson & Tregenza 2005, Tuda 2007). Females cement the eggs to the surface of the bean and approximately 4-5 days later (at 28°C) they hatch and the first instar larvae burrow into the seed (Fox 1993). Development and pupation takes place within the bean and emerging adults require neither food nor water for reproduction (Fox 1993). These factors make them particularly suitable for controlled, laboratory based experiments.

All beetles used in the following tests came from cultures maintained in glass jars containing approximately 100 black-eyed beans, *Vigna unguiculata*. The jars were kept in a 29˚C, 50% relative humidity climate chamber with a 12:12h light: dark cycle. Each jar contained only one introgression line. Individual beans were isolated in Eppendorf tubes hatching to ensure virginity.

Construction of Introgression lines

Nine introgression lines, lines containing individuals with three different nuclear haplotypes and three mitochondrial haplotypes, of *C. maculatus* were established in the lab. These nine lines where later tested for differences in behaviours and life history traits. The results were later analyzed in order to see the significance of the mitochondrial genome by itself and in interaction with the nuclear genome.

To set up these lines, outbreed stocks of three C*. maculatus* populations were used to generate 9 mito-nuclear introgression lines, combinations of distinct mitochondrial and nuclear lineages (Table 1). The three populations that were used originated from Brazil [Br], California [Ca] and Yemen [Ye]. A single virgin female from each of the three stocks was first mated to a male from the same stock. Three of the full-sib virgin daughters were later placed in a jar containing 150 beans with three males from one of the three stocks. These three females were effectively mitochondrial "Eves". The same procedure was implemented to virgin females from each outbred stock. In each following generation, virgin females from each of the nine lines were backcrossed with outbred males from the same stock population as their fathers. This backcrossing was used to dissociate the nuclear genome of the female, replacing it with the one originating from the male stock population. This repeated backcrossing was performed 13 consequent generations. Even if no infections of the mitochondrial inherited bacteria *Wolbachia* have been detected in *C. maculatus* (Tuda *et al.* 2006), treatment with tetracycline hydrochloride at generation 12 to rid possible bacterial infections was executed. The repeated backcrossing would, without any strong selection for a certain cytonuclear combination within lines during introgression (Arnqvist et al. 2010), result in >99.9% of the original nuclear genome of each female line being replaced. The result would be expression of the three mitochondrial genomes with one of each nuclear genome as shown in table 1. For all introgression lines there were three replicates (three different jars) and I used beetles from two generations.

Table 1. ´Mito-nuclear´ introgression lines generated from three mitochondrial genomes (mt) and three nuclear genomes (n). Mitochondrial genes are inherited maternally and introgressed with nuclear DNA from males of the stock populations.

	mt Br	mt Ca	mt Ye
n Br	n Br × mt Br	n Br × mt Ca	n Br × mt Ye
n Ca	n Ca × mt Br	n Ca × mt Ca	n Ca × mt Ye
n Ye	n Ye × mt Br	n Ye × mt Ca	n Ye × mt Ye

Test 1- life span

All individuals used were sampled from the nine introgression lines constructed. The Eppendorf tubes, that each contained one bean, was checked twice daily (1000h & 1700h ± 1h) for emerging beetles and dead ones. Once an individual had emerged the bean was removed and the time of emergence was recorded. The same procedure was used in order to determine time of death generating life span data.

Test 2- activity

Beetles that had emerged during the night were introduced to an activity test on their second day. The test was performed during the afternoon (i.e. 1300 – 1800h). This resulted in the beetles being 27 till 39h old when the test was carried out. The activity test was constructed as following: A beetle was placed in a Petri dish (diameter 88.6 mm and height 10 mm) that was divided into nine sections (one circular in the middle and eight surrounding). The dish, with a lid, was shaken for three seconds to scare the beetle and trigger any potential stress or anti-predator response. The test started as soon as the dish had been shaken, and lasted for 10 minutes. The initial position of the beetle was noted and every time the beetle changed a section, it was recorded. Individual latency to walk and total number of section changes was recorded. In addition, every 30 seconds each beetle was assigned one out of three “activities”; resting, moving (any movement except walking) and walking. These was then assigned a score (resting = 0, moving = 1 and walking = 3) and every individual got a total score. This response is later called ‘Resting, moving and walking´.

Test 3-adult weight at emergence

Beetles were weighed within 30 minutes after collection. This was done to the nearest microgram using a Sartorius Genius ME 235. All individual weighing was done twice and the mean value was used when the statistical analyses were carried out.

Statistical analysis

I analyzed the data from the three tests using one Generalized Linear Mixed Model (GLMM) for each response. Response variables were: **(i)** 'lifespan' (i.e. number of days a beetle lived), **(ii)** 'adult weight at emergence' **(iii)** 'latency to walk' (i.e. the latency until the beetles started walking in the activity test), **(iv)** 'activity' (i.e. number of section lines crossed in 10 minutes), **(v)** ´Resting, moving and walking' (i.e. the action taking place every 30 seconds with its assigned score. All models had nDNA haplotype, mtDNA haplotype and 'sex' (i.e. male or female) as fixed effects, and 'replicate' (i.e. 1-3) and 'generation' (i.e. 1-2) as random effects. The interaction between nDNA and mtDNA was added; however, whenever the interaction was non-significant ($p > 0.05$) the interaction was removed from the model. Latency to walk was used as a continuous co-variate when analyzing `activity´ and ´resting, moving and walking´, since it had an effect in both models. The distribution of the different response variables and their residuals distribution can be viewed in table 2. All GLMMs were performed in SAS 9.2, version 2002-2008.

Table 2. Distribution of the responses and their residuals distribution, investigating variation of behavior and life history traits in Seed beetles (*Callosobruchus maculatus*).

Response	Distribution
Resting, moving and walking	Gaussian
Activity	Poisson
Latency to walk	Poisson
Life span	Gaussian
Adult weight at emergence	Gaussian

RESULTS

Test 1- life span

Life span was clearly affected by the sex ($F_{1,339}$ = 179.99, P = <0.0001), and females lived longer than males (Females: 21.5058 ± 1.9434, Males: 14.8152 ± 1.9312). When investigating both sexes separately, variation in lifespan was explained by nuclear background (Females: $F_{2,140}$ = 6.91, P = 0.0014; Males: $F_{2,194}$ = 4.61, P = 0.0110). Female life span was in addition influenced by the nuclear and mitochondrial haplotype interaction ($F_{4,136}$ = 2.60, P = 0.0388). However this was not the case in males ($F_{4,190}$ = 0.17, P = 0.9542).

Test 2- activity

Activity

Activity was influenced by sex ($F_{1,371}$ = 23.04, P <0.0001) and males were more active than females (Females: 2.7425 ± 0.2628, Males: 3.1137 ± 0.2597). When each sex was analyzed separately, the interaction between nuclear and mitochondrial haplotypes affected activity levels of beetles, and so did the mtDNA on its own (Table 3).

Table 3. Factors affecting activity of Seed beetles (*Callosobruchus maculatus*) when analyzed in a Generalized Linear Mixed Model.

Effect	*Num df*	*Den df*	*F*	*P*
Females				
nDNA	2	168	2.26	0.1079
mtDNA	2	168	2.96	0.0547
mtDNA*nDNA	4	168	5.25	0.0008
Latency to walk	1	168	30.66	0.0107
Males				
nDNA	2	193	1.43	0.2415
mtDNA	2	193	3.78	0.0245
mtDNA*nDNA	4	193	2.47	0.0463
Latency to walk	1	193	26.83	<0.0001

Resting, moving and walking

Again, sex had an effect on the combined score of resting, moving and walking ($F_{1,371}$ = 25.12, P = <0.0001) and males showed more activity (Females: 23.6715 ± 3.4121, Males: 30.2691 ± 3.3846) However, when each sex was analyzed separately, the interaction between mtDNA and nDNA had an effect on resting, moving and walking for both sexes (Table 4), and so did latency to walk.

Table 4. Factors affecting the combined score of resting, moving and walking of Seed beetles (*Callosobruchus maculatus*) when analyzed in a Generalized Linear Mixed Model.

Effect	*Num df*	*Den df*	*F*	*P*
Females				
nDNA	2	168	1.69	0.1871
mtDNA	2	168	0.78	0.4617
mtDNA*nDNA	4	168	4.31	0.0024
Latency to walk	1	168	26.41	<0.0001
Males				
nDNA	2	193	1.32	0.2697
mtDNA	2	193	1.57	0.2107
mtDNA*nDNA	4	193	5.34	0.0004
Latency to walk	1	193	50.81	<0.0001

Test 3-Adult weight at emergence

Adult weight at emergence was affected both by the interaction between mtDNA and nDNA ($F_{4,329}$ = 2.53, P = 0.0407) and sex ($F_{1,329}$ = 269.99, P = <0.0001). Females weighed more when emerging compared to males (Females: 5.3293 ± 0.06259, Males: 3.9499 ± 0.05930). However, in separate models for each sex, the interaction between nuclear and mitochondrial haplotypes was no longer significant, but in both females and males, nDNA haplotype had an effect on adult weight at emergence (Table 5).

Table 5. Factors affecting adult weight at emergence in Seed beetles (*Callosobruchus maculatus*) when analyzed in a Generalized Linear Mixed Model.

Effect	*Num df*	*Den df*	*F*	*P*
Females				
mtDNA	2	155	2.18	0.1161
nDNA	2	155	4.38	0.0142
Males				
mtDNA	2	174	0.49	0.6141
nDNA	2	174	4.77	0.0096

DISCUSSION

I used nine introgression lines generated from three nuclear and three mitochondrial haplotypes. I tested these nine lines for behaviours (activity and `resting, moving and walking´) as well as the two life history traits: life span and adult weight of emergence. I found that all responses had a genetical control from either nDNA, mtDNA or the interaction between them. In addition, all behaviours and life history traits investigated here were affected by the sex (i.e male or female) of the beetles. For life history traits, this confirms older studies showing that female *C. maculatus* both lives longer and are bigger than males (Utida 1972, Tuda 2007).

The life history traits investigated here (life span and adult weight of emergence) both showed an nDNA link, a link that was not apparent in the behaviours. To find a nuclear association in regards to the life history traits is not surprising since the different nuclear haplotypes all had different origins (Yemen, Brazil and California). Differences in life history traits between populations originating from different environments are commonly found (Stamps 2007, Wolf et al. 2007), and is likely to be caused by differences in selective pressure for the different life history traits.

In a recent study, Innocenti *et al.* (2011) showed that mitochondrial polymorphisms inflict sexual asymmetry in patterns of nuclear gene expression between female and male *Drosophila melanogaster*. It was revealed that mitochondrial polymorphism had major effects on nuclear gene expression in males, but few effects were found in females. Whether this has an indirect effect on behavior or life history traits remain to be tested, but I have here revealed that altered life spans was evident in females from mito-nuclear interactions but not in males. This suggests a possibility that genes on the nuclear and mitochondrial genome, which interact in their expression, may be harboring female-specific mutations in *C. maculates*. This in turn might affect life span and possibly other life history traits. Frank & Hurst (1996) brings forth population genetic models that suggest that mtDNA mutations that are deleterious to one sex but not to the other can be maintained at low frequencies. The efficiency of selection acting to rid such mutations would be dampened due to the strict maternal transmission and lack of recombination in the mitochondrial genome (Innocenti *et al.* 2011). Even if affected life span is not deleterious, the same theory could still be applied and therefore be a possible explanation to why asexual asymmetry in life span occur in *C. maculates*.

Adult weight of emergence in *C. maculatus* was not affected by nuclear background, but from in an interaction with mtDNA. If the adult weight of emergence is under some mitochondrial control as suspected, this might further conclude that growth rate experiences a similar relation. Since size is partly growth rate dependent (Mirth and Riddiford 2007), an increase in growth rate would lead to a bigger size. Different sizes within a population could mean numerous advantages and disadvantages in situations, for example competition, mating and predator avoidance. It is also reasonable that size differences within a species do owe some explanation to the ATP producing efficiency (i.e metabolic rate). Therefore, it does not come as a surprise that the mtDNA does affect adult weight of emergence and perhaps growth rate.

It has earlier been shown that there is a connection between mtDNA and metabolic rate (Tielemann *et al.* 2009, Arnqvist *et al.* 2010), but to further link this to behavior has remained unverified. I have here demonstrated that the mtDNA, by itself, inflict an effect on activity. The rather logical procession that variation in mtDNA imposes variation in metabolism leading further to variation in behaviours linked to metabolism is likely to exist.

This procession might therefore be of importance in the further investigation of mitochondrial DNA impact on animal ecology and ethology. Not only do these results add additional verification strengthening the view of mtDNA being under influence of adaptive selection. It also portrays an intriguing picture of the importance mitochondrial DNA might have on the evolution of variation in consistent behaviours, or that of behaviour with limited plasticity (i.e personalities). If activity and the life history traits included here is under some mitochondrial control as the results show, it would mean that due to the strict maternal inheritance and the lack of recombination, there is less for selection to act upon.
Due to the strict maternal transmission and lack of recombination in mtDNA, a trait or behavior linked to mtDNA would result in less alleles within a population for that specific trait or behaviour. A non-additive genetic basis for variance and therefore a more complex genetic control would make the specific trait more stable. In other words, a trait or behavior would be less ready to evolve and would therefore be more consistent over time than if genetic variation was additive.

An important aspect of this is that activity seems to be under genetical control, the same genetical control that generates most of the ATP and thereby most of the energy available for animals to use. If ATP production and activity is genetically linked it would mean that activity has relevance for other important characteristics involved in the use of ATP, such as reproduction. Furthermore, if activity and the rate of ATP production are selected for together, it is most likely that more behaviours would be selected for or against indirectly as most behaviours are ATP dependent. This complex selection pattern in which behaviours are linked to one another could then work to maintain variation within a population. This would then be of great interest in research regarding behavioural traits and personalities.

To summarize it all: I have found that the strict maternally transmitted mtDNA has through interaction with the nuclear DNA, but also by itself, has a genetical link to activity and two life history traits. Not only does this further suggest that the mitochondrial DNA is subject to selection but it also suggests that there is a link between ATP production, activity and possibly life history traits. For further investigations regarding mtDNAs effect on behaviours and life history traits, a study with the aim to link metabolic rate and mtDNA haplotypes would be of interest. To test the theory of mtDNA, metabolic rate and activity being linked together would be a natural progression to shed further light on what still is a much unknown area. An even more molecular approach would be the use of insertional mutagenesis in for example *Drosophilla melanogaster,* as mutant *Drosophilla melanogaster* containing P-elements is commonly used in genetical research. This method would allow assigning a more detailed picture a certain gene of interest may have for the behaviour tested.

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