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Complex mitonuclear interactions and metabolic costs of mating in male seed beetles

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Abstract

The lack of evolutionary response to selection on mitochondrial genes through males predicts the evolution of nuclear genetic influence on male-specific mitochondrial function, for example by gene duplication and evolution of sex-specific expression of paralogs involved in metabolic pathways. Intergenomic epistasis may therefore be a prevalent feature of the genetic architecture of male-specific organismal function. Here, we assess the role of mitonuclear genetic variation for male metabolic phenotypes [metabolic rate and respiratory quotient (RQ)] associated with ejaculate renewal, in the seed beetle *Callosobruchus maculatus*, by assaying lines with crossed combinations of distinct mitochondrial haplotypes and nuclear lineages. We found a significant increase in metabolic rate following mating relative to virgin males. Moreover, processes associated with ejaculate renewal showed variation in metabolic rate that was affected by mitonuclear interactions. Mitochondrial haplotype influenced mating-related changes in RQ, but this pattern varied over time. Mitonuclear genotype and the energy spent during ejaculate production affected the weight of the ejaculate, but the strength of this effect varied across mitochondrial haplotypes showing that the genetic architecture of male-specific reproductive function is complex. Our findings unveil hitherto underappreciated metabolic costs of mating and ejaculate renewal, and provide the first empirical demonstration of mitonuclear epistasis on male reproductive metabolic processes.

Introduction

Mitochondria are vital to cellular metabolism and are the most important provider of energy through oxidative phosphorylation (OXPHOS) pathway. The OXPHOS pathway is comprised of products of both mitochondrial DNA (mtDNA) and nuclear-encoded genes, which also regulate mtDNA transcription and replication (Levin *et al.*, 2014). An increasing number of studies point to an important role for mitochondrial function in both male and female reproduction (e.g. Ramalho-Santos *et al.*, 2009; Rajender *et al.*, 2010; Yee *et al.*, 2013). However, the maternal inheritance of mtDNA means that selection on mtDNA in males does not generally yield an evolutionary response (Frank & Hurst, 1996; Gemmell *et al.*, 2004), except under restricted conditions (Wade & Brandvain, 2009). This predicts the evolution of male-specific (e.g. in male reproductive tissues) regulation of nuclear-encoded genes with mitochondrial function (Meiklejohn *et al.*, 2007; Gallach & Betran, 2011; Wade, 2014). Recent studies in *Drosophila* provide support for this, showing Y-linked regulation of testis-specific expression of OXPHOS genes (Lemos *et al.*, 2008; Sackton *et al.*, 2011; Branco *et al.*, 2013) with consequences on male fertility (Yee *et al.*, 2015), the evolution of testis-specific expression of nuclear OXPHOS gene duplicates (Gallach *et al.*, 2010) and pronounced transcriptional interactions between mtDNA and nuclear metabolic genes in male reproductive tissues (Innocenti *et al.*, 2011). Male-specific mitonuclear genetic effects should therefore be pronounced, and have indeed been shown to affect male fertility in hares (Smith *et al.*, 2010) and humans (Holyoake *et al.*, 2001) and sperm traits in beetles (Dowling *et al.*, 2007b) and chicken (Froman & Kirby, 2005).

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Considering the central role that mitonuclear genetic variation has in metabolic processes (Arnqvist *et al.*, 2010; Ballard & Melvin, 2010) and the male-specific regulation of some of the genes involved (Lemos *et al.*, 2008; Gallach *et al.*, 2010; Innocenti *et al.*, 2011), the metabolism of male reproduction should be a hot spot for epistasis arising from mitonuclear genetic variation (Beekman *et al.*, 2014). One of the two main aims of the current study was to assess whether mitonuclear genetic effects are indeed stronger for those metabolic processes that are engaged after mating in males, compared to more general metabolic processes. Post-mating metabolism powers physiological processes in males that involve the renewal of the ejaculate by active biosynthesis in testes and reproductive accessory glands.

The metabolic processes involved in ejaculate production should carry a cost to males in terms of increased energetic expenditure. Such costs related to reproduction are central to the evolution of mating systems and life-history strategies (Trivers, 1972; Emlen & Oring, 1977; Parker, 1979; Wade & Shuster, 2003). Across taxa, females invest far more into gamete biosynthesis compared to males (Hayward & Gillooly, 2011). Yet, the cost of producing entire ejaculates may be significant (Dewsbury, 1982; Hayward & Gillooly, 2011; Scharf *et al.*, 2013). For example, males of many taxa become ejaculate depleted when engaging in multiple successive matings (Birkhead & Fletcher, 1995; Olsson *et al.*, 1997; Arnqvist & Danielsson, 1999; Preston *et al.*, 2001; Ambriz *et al.*, 2002; Rigaud & Moreau, 2004; Damiens & Boivin, 2006; Rönn *et al.*, 2008; Sirot *et al.*, 2009; Doyle, 2011), which limits male reproductive success. Different ejaculate traits may also trade-off with one another (Pitnick, 1996; Oppliger *et al.*, 1998; Immler *et al.*, 2011) and the cost of mating can limit investment into other male life-history traits (e.g. Cordts & Partridge, 1996; Mappes *et al.*, 1996; Kotiaho & Simmons, 2003; Martin & Hosken, 2004; Paukku & Kotiaho, 2005; Ferkau & Fischer, 2006; Oliver & Cordero, 2009; South *et al.*, 2009; Salehialavi *et al.*, 2011). Although reproductive costs related to ejaculate production are well documented, our understanding of their origin is still limited: comparative data suggest that energetics poses global constraints on male gamete production (Hayward & Gillooly, 2011), but very few studies have attempted to estimate the energetic expenditure of ejaculate production empirically (Thomsen *et al.*, 2006). Our second main aim was to provide such an estimate.

Here, we integrate whole-organism microrespirometric phenotyping with a fully crossed introgressive breeding design to (i) estimate the degree to which male metabolism associated with ejaculate production increases energetic costs, and to (ii) test the prediction that mitonuclear genetic effects contribute more to variation in metabolic phenotypes in recently mated males compared to virgin males in the seed beetle *Callosobruchus maculatus*. We assay both metabolic rate and metabolic substrate usage [i.e. the respiratory quotient (RQ)]. Virgin males are ready to mate immediately upon eclosion and transfer on average 5–6% of their body weight in their first mating, after which the ejaculate is renewed during some 20–30 h (Fox *et al.*, 1995; Rönn *et al.*, 2008). We experimentally estimate the metabolic cost of post-mating processes including ejaculate renewal by comparing the energetic expenditure of mated males that are actively renewing their ejaculate with virgin males that are not. To provide further insights into the consequences of this energetic expenditure, we ask whether the energy spent following the first mating affects the size of the second ejaculate and whether this relationship differs across mitonuclear genotypes. We predict that increased energetic expenditure during ejaculate renewal results in a larger ejaculate.

Materials and methods

Study species

The seed beetle *C. maculatus* is a pest of legume plants and occurs in subtropical and tropical regions throughout the world. It is facultatively aphagous; adults can complete their adult reproductive cycle with the resources acquired during the larval stage (Fox, 1993). Both sexes can mate multiply and copulate right after emergence. The size of the male ejaculate influences both male fertilization success and female fecundity (Fox, 1993; Eady, 1995; Savalli & Fox, 1999a; Arnqvist *et al.*, 2004). Costs of male ejaculate production in *C. maculatus* have previously been inferred from the fact that males that mate more often (Paukku & Kotiaho, 2005) or for longer (Brown *et al.*, 2009) suffer reduced lifespan. Further, mitonuclear genetic effects have been documented for metabolic rate in pupae (Arnqvist *et al.*, 2010) and juvenile growth rate in both males and females (Dowling *et al.*, 2007a).

Mitonuclear lines

To disentangle the effects of mitochondrial and nuclear genetic variation as well as their interaction, we used lines that carry distinct mitochondrial genomes introgressed into different nuclear genetic backgrounds. Three outbred stock populations of *C. maculatus* [Brazil (BRA), California (CA) and Yemen (YEM)] were used to generate nine fully crossed combinations of distinct cytoplasmic and nuclear lineages. These populations carry different mtDNA haplotypes, based on two diagnostic markers (1005-bp fragment of *cytochrome oxidase subunit I* [*COI*] and 473-bp fragment of cytochrome *b* [Cyt-*b*]) that show both synonymous and non-synonymous polymorphisms (Arnqvist *et al.*, 2010;

Kazancioglu & Arnqvist, 2013). The three populations used were selected from a larger set of potential populations, as previous research has shown that mitonuclear genetic variation across these populations has phenotypic effects (Dowling *et al.*, 2007a, 2010; Arnqvist *et al.*, 2010). A single randomly selected virgin female from each of the three stocks was first mated to a randomly selected male from the same stock population. Full-sib virgin daughters of these 'mitochondrial Eves' were then mated to males from either of the three stock populations, in all nine possible combinations. Each of the nine mitonuclear cross-types was replicated twice, starting with a different 'mitochondrial Eve' female (but carrying the same mtDNA haplotype as the other replicate 'Eve' in a given cross), resulting in a total of 18 introgression lines. Within each cross, in every subsequent generation, the virgin daughters were backcrossed to males randomly sampled from their father's population thus disassociating the maternal mitochondrial genome from the nuclear genome that it was originally coexpressed with. Backcrossing was repeated for 15 generations, which in theory means that more than 99.99% of the original nuclear genome was replaced with the nuclear genome of the paternal population (assuming lack of strong selection on specific mitonuclear allelic combinations during the introgression procedure). The lines were backcrossed once again to the outbred stock populations at generation 17. Mitochondrial genetic integrity of our introgression lines was validated immediately after the creation of the lines (Kazancioglu & Arnqvist, 2013) as well as after our experiments, by amplification and sequencing of a diagnostic mtDNA gene (*COI*). This also verified that the two replicate lines for each cross-type carried the same mtDNA haplotype. After the 17th generation, the 18 mitonuclear lines were maintained as separate populations (> 400 individuals per population) in large glass jars seeded with 100 g of black-eyed beans, *Vigna unguiculata*, at 29 °C, with a relative humidity of 50% and at a 12:12 h light:dark cycle. No food or water was provided for the adults. A more detailed description of the construction of these lines can be found in Kazancioglu & Arnqvist (2013).

Maternally inherited cytoplasmic bacteria can potentially confound the results of experiments that aim to investigate mitochondrial genetic effects. Here, we term and interpret the cytoplasmic effects observed as being mitochondrial in origin based on the following three facts. First, infections with endosymbiotic bacteria (e.g. Wolbachia) have been carefully screened for in many *C. maculatus* populations, including those used here, but have never been detected (Kondo *et al.*, 1999; Tuda *et al.*, 2006; Kageyama *et al.*, 2010). Second, to preclude the possibility that our introgression lines may nevertheless have harboured cytoplasmic bacterial infections, we treated all introgression lines with an effective antibiotic treatment between generations 13 and 14 (see Kazancioglu & Arnqvist, 2013). Third, mtDNA haplotypes that are genetically more distinct are also more dissimilar in terms of their metabolic phenotype in *C. maculatus*, in line with a mitochondrial origin of cytoplasmic effects (Arnqvist *et al.*, 2010).

Experimental procedures

Mating treatment

The entire experiment was repeated in three subsequent generations (i.e. experimental blocks). For collection of virgins, we isolated individual beans in chambers from which the hatching adults were collected after 21 days from the onset of egg laying. Virgins were isolated in 1.5-mL Eppendorf tubes (with lids punctured for air) and used for the experiment when 24–48 h old.

To test whether mating affects male metabolic rate and whether this depends on mitonuclear genetic variation, we carried out the following experimental procedures. In total, 540 males from the 18 introgression lines were used in the experiment. To improve the precision of the metabolic rate assay, males belonging to the same line were assayed in groups of three. We measured metabolic rate in two types of males: virgins and recently mated. We recorded the body mass of each male triad to the nearest 0.00001 g (Sartorius® Genius ME 235P, Sartorius AG, Goettingen, Germany). For the mated treatment, the males in each triad were separated after first being weighed and were paired individually with virgin females from the same line. After mating was observed, each triad was re-grouped and weighed again. The difference in body weight immediately before and after mating was taken as the ejaculate weight (Edvardsson & Tregenza, 2005; Rönn *et al.*, 2008). We recorded the copulation duration in each mating as the time from the moment males finished antennal display and leaned backwards while mounting the female, until the separation of the pair [mean ± SE copulation duration (s): 406 ± 10.6]. Immediately after the second weighing, each triad was placed into a respirometry chamber overnight (for 17 h) to allow appropriate time for ejaculate replenishment. On the following morning, each triad was weighed again and the males were re-mated to new virgin females. Their second copulation duration was recorded [mean ± SE (s): 532 ± 10.8] and post-mating weight measured again for each triad, yielding a measure of the weight of the second ejaculate that was formed during the time in which metabolism was recorded.

Males in the virgin treatment were also paired individually with virgin females (from the same line) in precisely the same manner and were allowed to court, but mating was interrupted when males attempted to mount the female and pairs were separated. This was carried out to control for any lasting physical effort of courtship. Again, males in each triad of virgin males were then re-grouped, weighed and placed into a

respirometry chamber in the same manner as for males in the mated treatment. The order in which each line was subjected to the two treatments (virgin/mated) was randomized within and between the experimental days.

Metabolic rate measurements

Metabolic rate was measured using a Sable Systems (Las Vegas, NV, USA) flow-through respirometry system (Lighton, 2008) (see the Appendix S1 for technical details and calibration). Briefly, the respirometry system was set up in stop-flow mode (Lighton 2008), in which each chamber was sealed for a period of 67 min and then flushed for a period of 4 min. Each cycle (through all chambers) lasted for 71 min, and each measuring session lasted approx. 17 h (between 5 PM and 10 AM). This resulted in 14 readings of CO_2 and O_2 produced in each individual chamber, of which the first was discarded as a burn-in. Each respirometry chamber was placed in an activity detector (AD-2; Sable Systems) connected to a data acquisition interface (Quick-DAQ; National Instruments, Coleman Technologies, Newton Square, PA, USA), which uses reflective infrared light technology to provide a precise and continuous measure of locomotor activity of the subjects in each chamber during the entire session. One of the 16 chambers was left empty and was used as a baseline to control for any drift of the gas analysers during each session. Thus, each observation essentially consisted of the amount of CO_2 produced and O_2 consumed during 71 min by a triad of males and the total amount of activity performed during this time.

Statistical analysis and hypotheses

The amount of CO_2 produced was correlated with O_2 consumed ($r = 0.82$ across all cycles and sessions), and models using the two alternative measures of metabolic rate yielded qualitatively identical and quantitatively very similar results. Hence, we only present data for metabolic rate based on CO_2 below. We estimated the RQ ($RQ = CO_2$ produced/O_2 consumed) to test for putative changes in the use of metabolic substrates associated with any of the factors and variables measured here. The RQ = 1 is expected for pure carbohydrate oxidation whereas $RQ \approx 0.7$ is expected under pure lipid oxidation (Kleiber, 1961). We calculated the total sum of CO_2 produced by each individual during the whole observation period, to test for effects on the total energy spent during 17 h. We also calculated the average RQ per individual across the same time period.

Our inferential models consisted of a series of linear mixed-effect models, fitted using the package *lme4* (Bates & Maechler, 2012) implemented in R (version 3.0.1) (R Development Core Team, 2011). First, we investigated effects on the sum of CO_2 across both mated and virgin males (Table 1), for mated males alone (Table 2) and for virgins alone (Table S2). Second, we tested for effects on the mean RQ across all males (Table 1). Third, to test for patterns across time we used CO_2 as the response variable for all data (Table S1), for virgin males alone (Table S3) and for mated males alone (Table S4). Fourth, we tested for effects on the weight of the first (Table S5) and second (Table 3) ejaculates. All models included mtDNA haplotype, nDNA lineage, their interaction (the key factors of interest) and experimental block as fixed factors, body weight and activity as covariates. We also included a squared (i.e. quadratic) term for activity where this was significant. In addition, we tested for possible block interaction effects but found none and we therefore report all models in the tables without these interactions for simplicity. All models involving mated

Table 1 Mixed-model analyses of mitochondrial, nuclear, mitonuclear and mating treatment effects on metabolic phenotypes in *Callosobruchus maculatus*. $N = 155$.

	Metabolic rate			Respiratory quotient		
Fixed effects	X^2	d.f.	P	X^2	d.f.	P
Nuclear lineage	1.0	2	0.6010	0.5	2	0.7673
Mitochondrial lineage	1.9	2	0.3873	1.5	2	0.4738
Mitochondrial × nuclear	14.4	4	**0.0062**	4.4	4	0.2169
Experimental block	166.3	2	**< 0.0001**	4416	2	**< 0.0001**
Weight	111.0	1	**< 0.0001**	1.5	1	0.2171
Activity	56.7	1	**< 0.0001**	0.1	1	0.9070
Mating treatment	5.5	1	**0.0188**	2.8	1	0.0956
Mitochondrial × treatment	0.4	2	0.8182	0.2	2	0.8977
Nuclear × treatment	1.8	2	0.4031	0.3	2	0.8456
Mitochondrial × nuclear × treatment	3.5	4	0.4746	7.4	4	0.1166
Random effects		Variance (σ^2)				Variance (σ^2)
Line		4.393×10^{-19}				0.00
Residual		0.0001757				0.0002602

P-values <0.05 highlighted in bold.

Table 2 Mixed model of the effects of the weight of the first ejaculate, copulation duration, mitochondrial, nuclear and mitonuclear genotype on metabolic rate in mated males. $N = 77$.

Fixed effects	X^2	d.f.	P
Nuclear lineage	2.8	2	0.2470
Mitochondrial lineage	10.0	2	**0.0067**
Mitochondrial × nuclear	20.5	4	**0.0004**
Experimental block	175.3	1	**< 0.0001**
Weight after mating	77.7	1	**< 0.0001**
Activity	87.6	1	**< 0.0001**
First ejaculate weight	2.9	1	0.0904
First copulation duration	0.4	1	0.5495
Mitochondrial × 1st ejaculate	7.9	2	**0.0194**
Nuclear × 1st ejaculate	6.2	2	**0.0457**
Mitochondrial × nuclear × 1st ejaculate	22.7	4	**0.0001**
Mitochondrial × 1st copulation duration	0.5	2	0.7599
Nuclear × 1st copulation duration	9.7	2	**0.0077**
Mitochondrial × nuclear × 1st copulation duration	2.6	4	0.6244

Random effects	Variance (σ^2)
Line	1.472×10^{-20}
Residual	0.00009182

P-values <0.05 highlighted in bold.

Table 3 Mixed model of the effects of metabolic rate, mitochondrial, nuclear and mitonuclear genotype on variation in the weight of the second ejaculate. $N = 74$ male triads.

Fixed effects	X^2	d.f.	P
Nuclear lineage	35.1	2	**< 0.0001**
Mitochondrial lineage	25.5	2	**< 0.0001**
Mitochondrial × nuclear	25.2	4	**< 0.0001**
Experimental block	21.4	2	**< 0.0001**
Weight before 2nd mating	45.2	1	**< 0.0001**
Weight loss between 1st and 2nd mating	36.4	1	**< 0.0001**
Second copulation duration	0.09	1	0.7608
First ejaculate weight	0.20	1	0.6524
Nuclear × 1st ejaculate	8.04	2	**0.0180**
Mitochondrial × 1st ejaculate	1.33	2	0.5125
Mitochondrial × nuclear × 1st ejaculate	14.5	4	**0.0059**
Total CO_2	0.04	1	0.8363
Mitochondrial × CO_2	7.22	2	**0.0271**
Nuclear × CO_2	1.97	2	0.3735
Mitochondrial × nuclear × CO_2	2.33	4	0.6746

Random effects	Variance (σ^2)
Line	0.00
Residual	8.886×10^{-9}

P-values <0.05 highlighted in bold.

males also included ejaculate weight and copulation duration (as focal covariates), as well as their interactions with mtDNA haplotype and nDNA lineage, to test whether these might affect the metabolic variation during ejaculate renewal in a genotype-dependent manner. We also accounted for effects of additional model-specific covariates and interactions (see below and the Appendix S1), and the *P*-values are reported from full models. Line identity ($N = 18$) was always included as a random-effects factor to account for the fact that two replicate lines were used per mitonuclear combination. Further, in the models of metabolic rate and RQ over time (Table S1), male triad was treated as a subject and subject identity was included as a random-effects factor to account for the repeated measures taken over the thirteen cycles for each subject. Finally, we also explored whether mitonuclear combinations that share a more recent coevolutionary history (e.g. BRA × BRA) differ from those that do not (e.g. BRA × YEM) for both metabolic rate and RQ by fitting two models (for the sum of CO_2 and mean RQ with data on both mated and virgin males) analogues to the above, but where the mitochondrial lineage was replaced with a dichotomous 'coevolution' factor.

The significance of terms in the fitted models was assessed using analysis of variance with type III sums of squares tested with analyses of deviance based on the chi-square distribution, as implemented in the *car* package (Fox & Weisberg, 2011). Covariates in all models were standardized to a mean of zero and unit variance. Model fit was validated by visual inspection, and a few observations with an absolute value of the standardized residuals exceeding 3.0 were deemed outliers and omitted from further analysis. However, we note that this omission did not qualitatively alter our results.

Our main inferential aims were threefold. First, we tested the hypothesis that male metabolic rate is elevated after mating. Such an effect is likely to arise from mating directly, through biosynthetic costs of renewing ejaculates, or indirectly, through other hormonal and physiological processes such as mobilizing energy stores in the fat body, as the ejaculate in this species is very large and rich in proteins. Second, we compared the effects of mitochondrial and mitonuclear genetic variation on male metabolic rate in unmated males with that in mated males, with the explicit prediction that mitonuclear epistatic variation should have a larger effect in the latter than the former. This would be true under the assumption that copulation triggers male-specific germline and somatic biosynthetic processes (associated with ejaculate renewal) that are distinct from those engaged in virgin males, which should be more dominated by general metabolic processes. Third, for similar reasons, we tested the prediction that mitonuclear epistatic variation should affect the rate of ejaculate renewal, as manifested by the weight of the second ejaculate.

Results

The effect of mating on male metabolic rate

The average male metabolic rate was 0.021 μL of CO_2 min^{-1} mg^{-1}. Mated males exhibited a significantly

elevated post-copulatory metabolic rate compared to virgin males that were allowed to court females but were denied copulation ($\chi^2_1 = 5.5$, $P = 0.0188$, Table 1). This effect appeared after two measurement cycles and remained sizeable throughout the 17-h observation period following exposure to females (Fig. 1, Table S1). Overall, the metabolic output of once-mated males was 2.3% higher than that of virgin males (measured across cycles, corrected for variation in activity, body weight and experimental generation).

Genetic architecture of metabolism in mated and unmated males

The global models, involving both mated and unmated males, revealed significant mitonuclear genetic effects on male metabolism (see Tables 1 and S1). Neither metabolic rate nor RQ showed differences between virgin and mated males depending on the mitonuclear genotype; however, the treatment effect on RQ varied across time in a manner dependent on the mtDNA haplotype (Table S1, Fig. S1). We found no differences between native and novel mitonuclear combinations in either metabolic phenotype (CO_2: $\chi^2_1 = 0.5$, $P = 0.4981$; RQ: $\chi^2_1 = 1.0$, $P = 0.3281$), but the mating treatment effect on mean RQ depended upon the coevolutionary history of the mitonuclear genotypes (coevolution × treatment: $\chi^2_1 = 4.0$, $P = 0.0450$). To further characterize the difference between treatments, we analysed variation in metabolism in mated and unmated males separately. Importantly, this allowed us to control for variation in metabolism in mated males that was due to variation in ejaculate weight and copulation duration (Fig. 2).

We found only weak and statistically nonsignificant effects of mitochondrial lineage or mitonuclear combination on the metabolic rate of virgin males (Tables S2 and S3). The nuclear genetic lineage had a significant effect, which was primarily manifested as a difference across lineages in the pattern by which metabolic rate declined over time (i.e. a nuclear lineage × cycle interaction, Table S3). In contrast, metabolic rate in mated males was strongly affected by mitochondrial haplotype ($\chi^2_1 = 10.0$, $P = 0.0067$) as well as by mitonuclear genotype ($\chi^2_2 = 20.5$, $P = 0.0004$) (Table 2, Fig. 3), when controlling for variation in ejaculate weight and copulation duration. We also found a significant effect of mitochondrial haplotype on the rate at which metabolic rate declined over time ($\chi^2_{24} = 39.0$, $P = 0.0276$,

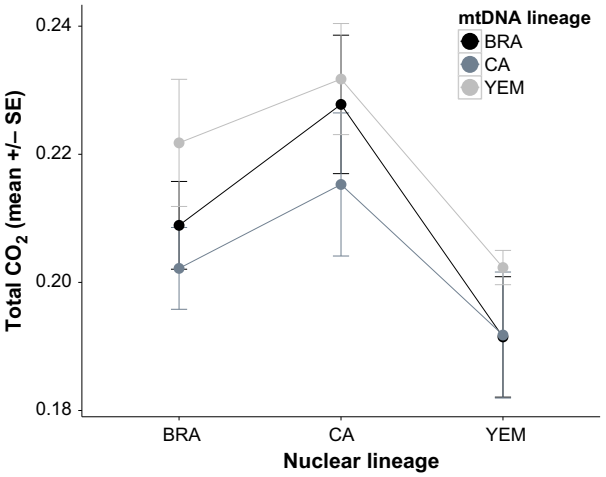

Fig. 2 The metabolic rate of mated males depends upon the mitonuclear genotype. Shown are activity corrected mean differences across the observation period, predicted from the model (Table 2).

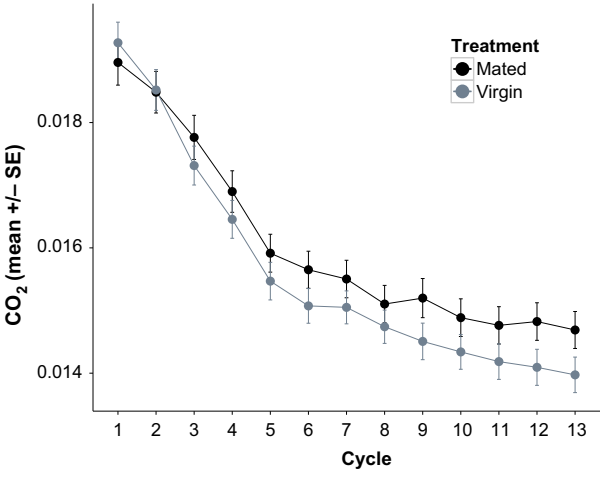

Fig. 1 Mating increases male metabolic rate across the observation period of 17 h (each cycle lasted 71 min). Shown are activity corrected mean differences, predicted from the model (Table S1).

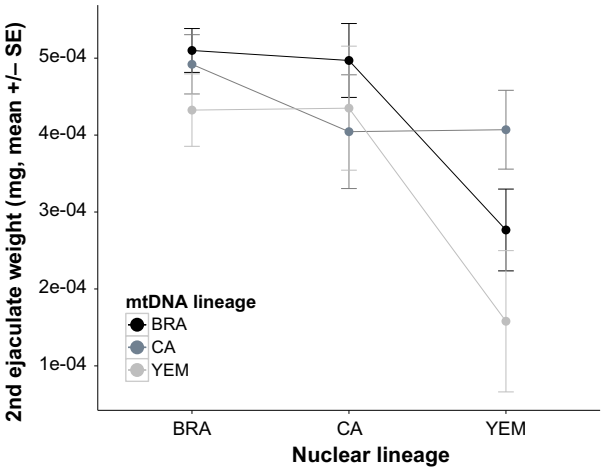

Fig. 3 The investment into the second ejaculate depends upon the mitonuclear genetic combination. Shown are mean differences predicted from the model (Table 3).

Table S4), which was not present in virgin males (Table S3).

We then performed focused tests of the prediction that ejaculate renewal exposes mitochondrial genetic variation in metabolic processes, rendering these genetic effects larger in mated compared to virgin males, by comparing effect sizes in models of mated vs. virgin males. Here, we used default (i.e. $\gamma/\alpha = 0.8$) directional variance ratio tests (Rice & Gaines, 1994). We found that the two common terms that include mitochondrial genetic effects explained more variance in metabolic rate in mated compared to unmated males, significantly so for one of the two terms (mitochondrial lineage: $F_{2,2} = 7.14$, $P_{dir} = 0.061$; mitochondrial × nuclear: $F_{4,4} = 4.18$, $P_{dir} = 0.048$).

Variation in the weight of the second ejaculate

As predicted, mitonuclear genetic variation affected the amount of ejaculate renewed (see Table 3 and Fig. 3). This was evident from the large main effects of both nuclear and mitochondrial lineage as well as from the significant mitonuclear interaction term. Moreover, the effect of the weight of the 1st ejaculate upon the weight of the 2nd differed across mitonuclear combinations. The total amount of energy spent during ejaculate renewal also affected the weight of the 2nd ejaculate, as predicted, but this effect differed across mitochondrial lines, as demonstrated by the significant interaction between mitochondrial line and the total amount of CO_2 produced in between matings (Table 3 and Fig. S2).

Discussion

We assessed whether ejaculate production is energetically costly in males and found a significant and non-trivial increase in metabolic rate in males following copulation. Moreover, we found support for the prediction that mitonuclear epistatic effects on metabolic processes are larger in mated males than in virgin males. Our results provide a series of novel insights. Below, we discuss the broader implications of metabolic costs of mating to males and of mating-specific mitonuclear effects on metabolic rate.

General life-history theory predicts a trade-off between allocation to current and future reproduction, and this should be true for male ejaculate allocation as well, assuming that ejaculates are costly to produce (Wedell *et al.*, 2002). Many components of courtship and mating carry costs to males (Scharf *et al.*, 2013), and several studies have documented trade-offs between male mating rate and other components of male fitness (e.g. Kotiaho & Simmons, 2003; Martin & Hosken, 2004; Paukku & Kotiaho, 2005; Simmons & Kotiaho, 2007; South *et al.*, 2009; Salehialavi *et al.*, 2011). However, the physiological origin of male reproductive costs remains poorly understood (Speakman, 2008; Scharf *et al.*, 2013). Our results suggest that such costs may derive in part from the energetic expenditure of ejaculate production. Although Dewsbury (Dewsbury, 1982) suggested that the 'physiological costs' of producing sperm and seminal fluid should be sizeable, we are only aware of one previous attempt to measure the energetic expenditure of ejaculate production empirically: Thomsen *et al.* (2006) estimated that ejaculate production makes up several per cent of the energy budget of male Japanese macaques during the breeding season. Our results are consistent with this, which suggests that gametogenesis and biosynthesis of other ejaculate components such as accessory gland proteins and peptides may typically be associated with sizeable metabolic costs. Although such costs should be general, their relative magnitude will depend on the size and composition of the ejaculate as well as on the energy budget of the taxa in question. We found that the metabolic expenditure is elevated by 2.3% after mating, which also temporarily increases the relative use of lipids as the primary metabolic substrate. The fact that RQ tended to be lower in mated males could reflect nutritional constraints but is also consistent with the hypothesis that lipids form a preferred respiratory substrate when performing metabolically demanding tasks in aphagous insects (Beenakkers, 1969), although we note that the strength of this effect varied across mitochondrial haplotypes and time after mating (Table S1, Fig. S1). The ejaculate of *C. maculatus* is large, constituting up to 6–8% of the male body weight (Rönn *et al.*, 2008; this study), and contains a rich and complex mixture of proteins and peptides (Goenaga *et al.*, 2015). Lipid energy resources available to males are limited and are obtained during larval feeding in this capital breeder. Although the metabolic costs documented here may not seem very large, we note that males can mate many times per day (Rönn *et al.*, 2008). The cumulated energy investment is thus very likely a major contributor to the decrease in male lifespan that follows from multiple mating in this species (Paukku & Kotiaho, 2005). Considering the male reproductive costs documented here, it is perhaps not surprising that male *C. maculatus* show prudent ejaculate allocation (Katsuki *et al.*, 2013) and that other seed beetle species show both male mate choice (Salehialavi *et al.*, 2011) and even sex-role reversal (Takakura, 1999; Fritzsche & Arnqvist, 2013).

Sequential mating reduces male ejaculate size in *C. maculatus* (Fox *et al.*, 1995; Rönn *et al.*, 2008), and the energy spent replenishing an ejaculate could therefore depend on the volume of the ejaculate transferred. We did detect an effect of a male's first ejaculate weight on the metabolic rate during ejaculate renewal, but this depended upon mitonuclear genotype (Table 2). This suggests that the overall metabolic costs of post-mating processes including ejaculate renewal correlate rather

poorly with ejaculate volume *per se*. The relative composition of the ejaculate, which is a complex cocktail of a very large number of proteins, peptides and carbohydrates along with sperm cells and water, shows marked differences across populations and may more closely reflect production costs. In addition, metabolic costs may arise from other hormonal and physiological changes that occur after mating. Ejaculate size is an important trait in terms of sexual selection that predicts male fertilization success in this species (Eady, 1995; Savalli & Fox, 1999b). We found variance in ejaculate size to be partly genetic in origin, but the patterns were not simple. For example, while nuclear genetic variation affected the size of the first ejaculate, this effect depended on copulation duration (Table S5). Similarly, there were both nuclear and mitochondrial genetic effects on the size of the second ejaculate, but these effects also interacted with each other, with the weight of the first ejaculate and with the total amount of CO_2 produced in between matings (Table 3). Mitochondrial haplotypes also differed in the rate at which the metabolic rate declined over time prior to the second mating (Table S4). This could reflect differences in the timing of ejaculate replenishment, and subsequently influence the patterns we see for the second ejaculate weight if some males were still renewing their ejaculate at the time of re-mating. These results point to a rather complex relationship between the metabolic cost of ejaculate production, male ejaculate size and the nuclear and mitochondrial genes that affect these phenotypic traits.

We also aimed to test the prediction that the metabolism of ejaculate production is disproportionally affected by mitonuclear epistasis. We found support for this prediction, in the form of larger mitochondrial genetic effects on metabolic rate in mated compared to unmated males. Yet, we note that the interaction between mitonuclear genotype and mating treatment on metabolic rate was not significant in our global model, and we suggest that this may in part derive from the fact that this model does not control for variance in metabolic rate among mated males that was associated with variation in ejaculate size and copulation duration. This interpretation is supported by the fact that the effect of mitonuclear genotype on metabolic rate was significantly larger in mated compared to virgin males when we controlled for these covariates.

Our findings have several implications. The fact that mating exposed mitonuclear epistasis on mating-related metabolic processes is largely consistent with recent studies showing that some male-specific metabolic pathways are regulated by nuclear-encoded genes with male-biased expression (Gallach *et al.*, 2010) or are under the control of Y-chromosome (Lemos *et al.*, 2008; Yee *et al.*, 2015). Such male-specific nuclear influences on mitochondrial function may have evolved in response to selective constraints on mtDNA due to its maternal inheritance (Gallach & Betran, 2011). In this regard, our findings are in line with an increasing number of reports that show mitonuclear epistasis for male reproductive traits. Innocenti *et al.* (2011) found that mitochondrial haplotype had major effects on male-specific gene expression of nuclear genes involved in metabolic and biosynthetic processes in testes and accessory reproductive glands of *Drosophila melanogaster*. Epistasis between mitochondrial and nuclear genes also compromises male fertility under sperm competition in *D. melanogaster* (Yee *et al.*, 2013). However, epistasis is not inevitable: for example, previous work on introgression lines in *C. maculatus* has shown main effects of mitochondrial haplotype variation on sperm length and sperm viability (Dowling *et al.*, 2007b). Future genomic work in *C. maculatus* will be aimed at identifying the genes responsible for the epistatic effects seen here and at assessing genetic variation and pleiotropic effects of these genes in both sexes.

The epistatic mitonuclear interactions exposed in our experiments shows that the compatibility of the allelic variants derived from the three different populations affect male-specific metabolic processes. Such effects should fuel selection on nuclear genes expressed in males for an optimal combination of alleles at those loci that interact with mtDNA (Wade, 2014). Different combinations may thus be favoured in divergent populations, and such co-adaptation can ultimately lead to genetic incompatibilities in population crosses (Gershoni *et al.*, 2009; Burton & Barreto, 2012; Trier *et al.*, 2014). However, we found no clear and consistent differences in either metabolic rate or RQ between native and novel mitonuclear combinations, but combinations that share a more recent history did tend to differ from other combinations with regard to the changes in respiratory substrate use that result from mating. We suggest that these patterns may reflect the complexity of these genetic effects across *C. maculatus* populations (Arnqvist *et al.*, 2010). Mitonuclear epistatic effects can involve a very large number of interactions between loci, and they interact not only with other aspects of the male-specific phenotype (i.e. ejaculate weight; Table 3) but also with environmental factors such as temperature (Arnqvist *et al.*, 2010; Hoekstra *et al.*, 2013) and food (Zhu *et al.*, 2014). Moreover, the fact that mitonuclear epistatic effects on metabolic rate are apparent in pupae (Arnqvist *et al.*, 2010) but not in virgin males (this study) suggests that they may differ over ontogeny. Thus, selection on mitonuclear genotypes seems to be entwined within a complex web of sex-, frequency-, environment-, genotype- and age-specific effects, which are likely to differ between populations (Rand *et al.*, 2001; Kazancioglu & Arnqvist, 2013). In addition, the relationship between metabolic phenotypes and fitness is likely sex- and environment-specific, as recently demonstrated in *C. maculatus* (Berger *et al.*, 2014). In the light of these complications, the lack of a clear phenotypic rank order in a particular environment between native and novel

mitonuclear interpopulation combinations is perhaps not that surprising (Arnqvist *et al.*, 2010).

Conclusions

We show that the physiological processes associated with ejaculate production are energetically costly to male beetles and we provide an experimental quantification of these male reproductive costs. Further, key aspects of the metabolic pathways that are engaged in males during ejaculate renewal are affected by mitochondrial and mitonuclear genetic variation, as revealed by a larger effect of mitonuclear genotype on metabolic rate in mated compared to unmated males. These findings are consistent with the predicted evolution of male-specific nuclear genetic effects on mitochondrial function.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Appendix S1 Stop-flow respirometry.

Table S1 Mixed model analysis of the effects of mating treatment, mitochondrial, nuclear and mitonuclear genotype on metabolic phenotypes in *Callosobruchus maculatus* males, across measurement cycles.

Table S2 Mixed model analysis of the mitochondrial, nuclear, mitonuclear genetic effects on metabolic rate in virgin males.

Table S3 Mixed model analysis of the mitochondrial, nuclear, mitonuclear genetic effects on metabolic rate in virgin males, across measurement cycles.

Table S4 Mixed model of the effects of the first ejaculate weight, mitochondrial, nuclear and mitonuclear effects on the metabolic rate in mated males, across measurement cycles.

Table S5 Effects of mitochondrial, nuclear, mitonuclear genetic variation on the first ejaculate weight.

Figure S1 Mating alters the respiratory quotient (RQ) throughout the observation time (of 17 h), but this depends on the mitonuclear lineage.

Figure S2 The relationship between post-mating CO_2 production and second ejaculate weight by mitochondrial lineage, as predicted by the model in Table 3.

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