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Citation for the original published paper (version of record):

Immonen, E., Rönn, J., Watson, C., Berger, D., Arnqvist, G. (2016)
Complex mitonuclear interactions and metabolic costs of mating in male seed beetles.
Journal of Evolutionary Biology, 29(2): 360-370
https://doi.org/10.1111/jeb.12789

Access to the published version may require subscription.

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

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Running title: Mitonuclear epistasis on male reproductive metabolism

Keywords: mitochondrial DNA, epistasis, metabolism, sex-specific selection, seed beetles, *Callosobruchus maculatus*

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 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 metabolism (metabolic rate and respiratory quotient) that was affected by mitonuclear interactions. Mitonuclear genotype and the energy spent during ejaculate production affected the weight of the ejaculate, but the strength of this effect varied across mtDNA 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 and Hurst 1996; Gemmell et al. 2004), except under restricted conditions (Wade and 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 and 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), 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 and Kirby 2005).

Considering the central role that mitonuclear genetic variation has in metabolic processes (Arnqvist et al. 2010; Ballard and 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 hotspot for epistasis arising from mitonuclear genetic variation (). One of the two main aims of the current study is 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 and Oring 1977; Parker 1979; Wade and Shuster 2003). Across taxa, females invest far more into gamete biosynthesis compared to males (Hayward and Gillooly 2011). Yet, the cost of producing entire ejaculates may be significant (Dewsbury 1982; Hayward and Gillooly 2011; Scharf et al. 2013). For example, males of many taxa become ejaculate depleted when engaging in multiple successive matings (Birkhead and Fletcher 1995; Olsson et al. 1997; Arnqvist and Danielsson 1999; Preston et al. 2001; Ambriz et al. 2002; Rigaud and Moreau 2004; Damiens and 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

88 male life-history traits (e.g. Cordts and Partridge 1996; Mappes et al. 1996;
89 Kotiaho and Simmons 2003; Martin and Hosken 2004; Paukku and Kotiaho
90 2005; Ferkau and Fischer 2006; Oliver and Cordero 2009; South et al. 2009;
91 Salehialavi et al. 2011). Although reproductive costs related to ejaculate
92 production are well documented, our understanding of their origin is still
93 limited: comparative data suggests that energetics poses global constraints on
94 male gamete production (Hayward and Gillooly 2011), but very few studies have
95 attempted to estimate the energetic expenditure of ejaculate production
96 empirically (Thomsen et al. 2006). Our second main aim is to provide such an
97 estimate.
98
99 Here, we integrate whole-organism microrespirometric phenotyping with a fully
100 crossed introgressive breeding design to (i) estimate the degree to which male
101 metabolism associated with ejaculate production increases energetic costs, and
102 to (ii) test the prediction that mitonuclear genetic effects contribute more to
103 variation in metabolic phenotypes in recently mated males compared to virgin
104 males in the seed beetle *Callosobruchus maculatus*. We assay both metabolic rate
105 and metabolic substrate usage (i.e., the respiratory quotient). Virgin males are
106 ready to mate immediately upon eclosion and transfer on average some 5-6% of
107 their body weight in their first mating, after which the ejaculate is renewed
108 during some 20-30 hours (Fox et al. 1995; Rönn et al. 2008). We experimentally
109 estimate the metabolic cost of post-mating processes including ejaculate renewal
110 by comparing the energetic expenditure of mated males that are actively
111 renewing their ejaculate with virgin males that are not. To provide further
112 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 and 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 and Kotiaho 2005) or for longer (Brown et al. 2009) suffer reduced life span. 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

In order to disentangle the effects of mitochondrial and nuclear genetic variation as well as their interaction, we used lines that carry distinct mitochondrial

138 genomes introgressed into different nuclear genetic backgrounds. Three outbred
139 stock populations of *C. maculatus* (Brazil [BRA], California [CA] and Yemen
140 [YEM]) were used to generate nine fully crossed combinations of distinct
141 cytoplasmic and nuclear lineages. These populations carry different mtDNA
142 haplotypes, based on two diagnostic markers (1005 bp fragment of *cytochrome*
143 *oxidase subunit I* [*COI*] and 473 bp fragment of *cytochrome b* [*Cyt-b*]) that show
144 both synonymous and non-synonymous polymorphism (Arnqvist et al. 2010;
145 Kazancioglu and Arnqvist 2013). The three populations used were selected from
146 a larger set of potential populations, as previous research has shown that
147 mitonuclear genetic variation across these populations have phenotypic effects
148 (Dowling et al. 2007a; Arnqvist et al. 2010; Dowling et al. 2010). A single
149 randomly selected virgin female from each of the three stocks was first mated to
150 a randomly selected male from the same stock population. Full-sib virgin
151 daughters of these "mitochondrial Eves" were then mated to males from either of
152 the three stock populations, in all nine possible combinations. Each of the nine
153 mitonuclear cross types was replicated twice, starting with a different
154 "mitochondrial Eve" female (but carrying the same mtDNA haplotype as the
155 other replicate "Eve" in a given cross), resulting in a total of 18 introgression
156 lines. Within each cross, in every subsequent generation, the virgin daughters
157 were backcrossed to males randomly sampled from their father's population
158 thus disassociating the maternal mitochondrial genome from the nuclear
159 genome that it was originally coexpressed with. Backcrossing was repeated for
160 15 generations, which in theory means that more than 99.99% of the original
161 nuclear genome was replaced with the nuclear genome of the paternal
162 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 and 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 100g 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 and 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 1.5ml Eppendorf tubes (with lids punctured for air) and used for the experiment when 24h-48h 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 (see below), 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). 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 and 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

212 the female, until the separation of the pair (mean ±SE copulation duration (sec):
213 406 ±10.6). Immediately after the second weighing, each triad was placed into a
214 respirometry chamber overnight (for 17 h) to allow appropriate time for
215 ejaculate replenishment. On the following morning, each triad was weighed
216 again and the males were re-mated to new virgin females. Their second
217 copulation duration was recorded (mean±SE (sec): 532±10.8) and post-mating
218 weight measured again for each triad, yielding a measure of the weight of the
219 second ejaculate that was formed during the time in which metabolism was
220 recorded.
221
222 Males in the virgin treatment were also paired individually with virgin females
223 (from the same line) in precisely the same manner and were allowed to court,
224 but mating was interrupted when males attempted to mount the female and
225 pairs were separated. This was done to control for any lasting physical effort of
226 courtship. Again, males in each triad of virgin males were then re-grouped,
227 weighed and placed into a respirometry chamber in the same manner as for
228 males in the mated treatment. The order in which each line was subjected to the
229 two treatments (virgin/mated) was randomized within and between the
230 experimental days.
231
232 *Metabolic rate measurements*
233 Metabolic rate was measured using a Sable Systems (Las Vegas, NV, USA) flow-
234 through respirometry system (Lighton 2008) (see the Online Appendix for
235 technical details and calibration). Briefly, the respirometry system was set up in
236 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. 17h (between 5PM and 10AM). 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, US), which uses reflective infra-red 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 analyzers during each session. Thus, each observation essentially consisted of the amount of CO_2 produced and O_2 consumed during 71 minutes 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 respiratory quotient (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 respiratory quotient RQ = 1 is expected for pure carbohydrate oxidation while RQ ≈ 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, in order to test for effects on the

262 total energy spent during 17h. We also calculated the average RQ per individual
263 across the same time period.
264
265 Our inferential models consisted of a series of linear mixed effect models, fitted
266 using the package *lme4* (Bates and Maechler 2012) implemented in R (version
267 3.0.1) (RDevelopmentCoreTeam 2011). First, we investigated effects on the sum
268 of CO_2 across both mated and virgin males (Table 1), for mated males alone
269 (Table 2) and for virgins alone (Table S2). Second, we tested for effects on the
270 mean RQ across all males (Table 1). Third, to test for patterns across time we
271 used CO_2 as the response variable for all data (Table S1), for virgin males alone
272 (Table S3) and for mated males alone (Table S4). Fourth, we tested for effects on
273 the weight of the first (Table S5) and second (Table 3) ejaculate. All models
274 included mtDNA haplotype, nDNA lineage, their interaction (the key factors of
275 interest) and experimental block as fixed factors, body weight and activity as
276 covariates. We also included a squared (i.e., quadratic) term for activity where
277 this was significant. In additiona, we tested for possible block interaction effects
278 but found none and we therefore report all models in the tables without these
279 interactions for simplicity. All models involving mated males also included
280 ejaculate weight and copulation duration (as focal covariates), as well as their
281 interactions with mtDNA haplotype and nDNA lineage, to test if these might
282 affect the metabolic variation during ejaculate renewal in a genotype-dependent
283 manner. We also accounted for effects of additional model-specific covariates
284 and interactions (see below and the Online Appendix) and the p-values are
285 reported from full models. Line identity (N=18) was always included as a
286 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 (on-line appendix), 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 and 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 metabolism 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. Such effects could be manifested in total CO_2 production and/or as alterations in RQ values, as changes in energetic requirements are often associated with changes in respiratory substrate and lipids accumulated during the larval stages form the primary respiratory substrate during active metabolism in seed beetles (Wightman 1978). 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 0.021µl of CO_2/min/mg. Mated males exhibited a significantly elevated postcopulatory metabolic rate compared to virgin males that were allowed to court females but were denied copulation (χ^2_1 = 5.5, P = 0.0188, Table 1). This effect appeared after two measurement cycles (142min) and remained sizeable throughout the 17 hour 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, 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). However, the mating treatment effect on RQ depended upon the coevolutionary history of the mitonuclear genotypes (coevolution x treatment: $\chi^2_1 = 4.0$, P = 0.0450). To further characterize the difference between treatments, we analysed variation in metabolic phenotypes 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.

We found only weak and statistically non-significant effects of mitochondrial lineage or mitonuclear combination on the metabolic rate of virgin males (Tables S2, 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 =

362 0.0276, Table S4), which was not present in virgin males (Table S3).

363

364 We then performed focused tests of the prediction that ejaculate renewal
365 exposes mitochondrial genetic variation in metabolic processes, rendering these
366 genetic effects larger in mated compared to virgin males, by comparing effect
367 sizes in models of mated versus virgin males. Here, we used default (i.e., γ/α =
368 0.8) directional variance ratio tests (Rice and Gaines 1994). We found that the
369 two common terms that include mitochondrial genetic effects explained more
370 variance in metabolic rate in mated compared to unmated males, significantly so
371 for one of the two terms (mitochondrial lineage: $F_{2,2}$ = 7.14, P_{dir} = 0.061;
372 mitochondrial × nuclear: $F_{4,4}$ = 4.18, P_{dir} = 0.048).

373

374 *Variation in the weight of the second ejaculate*

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

385

386

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 and Simmons 2003; Martin and Hosken 2004; Paukku and Kotiaho 2005; Simmons and 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 percent of the energy budget of male

412 Japanese macaques during the breeding season. Our results are consistent with
413 this, which suggests that gametogenesis and biosynthesis of other ejaculate
414 components such as accessory gland proteins and peptides may typically be
415 associated with sizeable metabolic costs. Although such costs should be general,
416 their relative magnitude will depend on the size and composition of the ejaculate
417 as well as on the energy budget of the taxa in question. We found that the
418 metabolic expenditure is elevated by 2.3 % after mating, which also temporarily
419 increases the relative use of lipids as the primary metabolic substrate. The fact
420 that RQ tended to be lower in mated males could reflect nutritional constraints
421 but is also consistent with the hypothesis that lipids form a preferred respiratory
422 substrate when performing metabolically demanding tasks in aphagous insects
423 (Beenakkers 1969), although we note that the strength of this effect varied
424 across mitochondrial haplotypes and time after mating (Table S1). The ejaculate
425 of *C. maculatus* is large, constituting up to 6-8% of the male body weight (Rönn et
426 al. 2008, this study), and contains a rich and complex mixture of proteins and
427 peptides (Goenaga et al. *submitted*). Lipid energy resources available to males
428 are limited and are obtained during larval feeding in this capital breeder.
429 Although the metabolic costs documented here may not seem very large, we note
430 that males can mate many times per day (Rönn et al. 2008). The cumulated
431 energy investment is thus very likely a major contributor to the decrease in male
432 lifespan that follows from multiple mating in this species (Paukku and Kotiaho
433 2005). Considering the male reproductive costs documented here, it is perhaps
434 not surprising that male *C. maculatus* show prudent ejaculate allocation (Katsuki
435 et al. 2013) and that other seed beetle species show both male mate choice

436 (Salehialavi et al. 2011) and even sex-role reversal (Takakura 1999; Fritzsche
437 and Arnqvist 2013).
438
439 Sequential mating reduces male ejaculate size in *C. maculatus* (Fox et al. 1995;
440 Rönn et al. 2008) and the energy spent replenishing an ejaculate could therefore
441 depend on the volume of the ejaculate transferred. We did detect an effect of a
442 male's first ejaculate weight on the metabolic rate during ejaculate renewal but
443 this depended upon mitonuclear genotype (Table 2). This suggests that the
444 overall metabolic costs of post-mating processes including ejaculate renewal
445 correlates rather poorly with ejaculate volume *per se*. The relative composition
446 of the ejaculate, which is a complex cocktail of a very large number of proteins,
447 peptides and carbohydrates along with sperm cells and water, shows marked
448 differences across populations and may more closely reflect production costs.
449 Additionally, metabolic costs may arise from other hormonal and physiological
450 changes that occur after mating. Ejaculate size is an important trait in terms of
451 sexual selection that predicts male fertilization success in this species (Eady
452 1995; Savalli and Fox 1999b). We found variance in ejaculate size to be partly
453 genetic in origin, but the patterns were not simple. For example, while nuclear
454 genetic variation affected the size of the first ejaculate, this effect depended on
455 copulation duration (Table S5). Similarly, there were both nuclear and
456 mitochondrial genetic effects on the size of the second ejaculate but these effects
457 also interacted with each other, with the weight of the first ejaculate and with the
458 total amount of CO_2 produced in between matings (Table 3). Mitochondrial
459 haplotypes also differed in the rate at which the metabolic rate declined over
460 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 phenotypes 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). Such male-specific nuclear influences on mitochondrial function may have evolved in response to selective constraints on mtDNA due to its maternal inheritance (Gallach and

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 *D. 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 and Barreto 2012; Trier et al. 2014). However, we found no clear and consistent differences in either metabolic rate or respiratory quotient between native and novel mitonuclear combinations, but combinations that share a more recent history did tend to differ from other combinations with

regards 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 and 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 light of these complications, the lack of a clear phenotypic rank order in a particular environment between native and novel mitonuclear inter-population 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.

Acknowledgments

We thank the GENCON lab group and David Rand for comments on a previous version of this manuscript. This study was financed by the European Research Council (GENCON AdG-294333) and the Swedish Research Council (621-2010-5266).

References

Ambriz, D., A. M. Rosales, R. Sotelo, J. A. Mora, A. Rosado, and A. R. Garcia. 2002. Changes in the quality of rabbit semen in 14 consecutive ejaculates obtained every 15 minutes. Arch Androl 48:389-395.

Arnqvist, G. and I. Danielsson. 1999. Postmating sexual selection: the effects of male body size and recovery period on paternity and egg production rate in a water strider. Behav Ecol 10:358-365.

Arnqvist, G., D. K. Dowling, P. Eady, L. Gay, T. Tregenza, M. Tuda, and D. J. Hosken. 2010. Genetic architecture of metabolic rate: environment specific epistasis between mitochondrial and nuclear genes in an insect. Evolution 64:3354-3363.

Arnqvist, G., T. Nilsson, and M. Katvala. 2004. Mating rate and fitness in female bean weevils. Behav Ecol 16:123-127.

Ballard, J. W. and R. G. Melvin. 2010. Linking the mitochondrial genotype to the organismal phenotype. Mol Ecol 19:1523-1539.

Bates, D. and M. Maechler. 2012. lme4: Linear mixed-effects models using S4 classes. R package version 0.999999-0. http://CRAN.R-project.org/package=lme4.

Beenakkers, A. M. 1969. Carbohydrate and fat as a fuel for insect flight: A comparative study. J Insect Physiol 15:353-361.

Berger, D., E. C. Berg, W. Widegren, G. Arnqvist, and A. A. Maklakov. 2014. Multivariate intralocus sexual conflict in seed beetles. Evolution 68:3457-3469.

Birkhead, T. R. and F. Fletcher. 1995. Depletion determines sperm numbers in male zebra finches. Anim Behav 49:451-456.

Bonduriansky, R. and S. F. Chenoweth. 2009. Intralocus sexual conflict. Trends Ecol Evol 24:280-288.

Branco, A. T., Y. Tao, D. L. Hartl, and B. Lemos. 2013. Natural variation of the Y chromosome suppresses sex ratio distortion and modulates testis-specific gene expression in Drosophila simulans. Heredity 111:8-15.

Brown, E. A., L. Gay, R. Vasudev, T. Tregenza, P. E. Eady, and D. J. Hosken. 2009. Negative phenotypic and genetic associations between copulation duration and longevity in male seed beetles. Heredity 103:340-345.

Burton, R. S. and F. S. Barreto. 2012. A disproportionate role for mtDNA in Dobzhansky-Muller incompatibilities? Mol Ecol 21:4942-4957.

Cordts, R. and L. Partridge. 1996. Courtship reduces longevity of male Drosophila melanogaster. Anim Behav 52:269-278.

Damiens, D. and G. Boivin. 2006. Why do sperm-depleted parasitoid males continue to mate? Behav Ecol 17:138-143.

Dewsbury, D. A. 1982. Ejaculate Cost and Male Choice. Am Nat 119:601-610.

Dowling, D. K., K. C. Abiega, and G. Arnqvist. 2007a. Temperature-specific outcomes of cytoplasmic-nuclear interactions on egg-to-adult development time in seed beetles. Evolution 61:194-201.

Dowling, D. K., U. Friberg, and J. Lindell. 2008. Evolutionary implications of non-neutral mitochondrial genetic variation. Trends in Ecology and Evolution 23:546-554.

Dowling, D. K., T. Meerupati, and G. Arnqvist. 2010. Cytonuclear interactions and the economics of mating in seed beetles. Am Nat 176:131-140.

595 Dowling, D. K., A. L. Nowostawski, and G. Arnqvist. 2007b. Effects of cytoplasmic
596 genes on sperm viability and sperm morphology in a seed beetle:
597 implications for sperm competition theory? J Evol Biol 20:358-368.
598 Doyle, J. M. 2011. Sperm depletion and a test of the phenotype-linked fertility
599 hypothesis in Gray Treefrogs (*Hyla versicolor*). Can J Zool 89:853-858.
600 Eady, P. E. 1995. Why do male *Callosobruchus maculatus* beetles inseminate so
601 many sperm. Behav Ecol Sociobiol 36:25-32.
602 Edvardsson, M. and T. Tregenza. 2005. Why do male *Callosobruchus maculatus*
603 harm their mates? Behav Ecol 16:788-793.
604 Emlen, S. T. and L. W. Oring. 1977. Ecology, sexual selection, and evolution of
605 mating systems. Science 197:215-223.
606 Ferkau, C. and K. Fischer. 2006. Costs of reproduction in male *Bicyclus anynana*
607 and *Pieris napi* butterflies: Effects of mating history and food limitation.
608 Ethology 112:1117-1127.
609 Fox, C. W. 1993. Multiple mating, lifetime fecundity and female mortality of the
610 Bruchid beetle, *Callosobruchus maculatus* (Coleoptera, Bruchidae). Funct
611 Ecol 7:203-208.
612 Fox, C. W., D. L. Hickman, E. L. Raleigh, and T. A. Mousseau. 1995. Paternal
613 Investment in a Seed Beetle (Coleoptera, Bruchidae) - Influence of Male
614 Size, Age, and Mating History. Ann Entomol Soc Am 88:100-103.
615 Fox, J. and S. Weisberg. 2011. An {R} Companion to Applied Regression. Sage.
616 Frank, S. A. and L. D. Hurst. 1996. Mitochondria and male disease. Nature
617 383:224.
618 Franklin, A. M., Z. E. Squires, and D. Stuart-Fox. 2012. The energetic cost of
619 mating in a promiscuous cephalopod. Biol Lett 8:754-756.
620 Fritzsche, K. and G. Arnqvist. 2013. Homage to Bateman: Sex roles predict sex
621 differences in sexual selection. Evolution 67:1926-1936.
622 Froman, D. P. and J. D. Kirby. 2005. Sperm mobility: phenotype in roosters (*Gallus*
623 *domesticus*) determined by mitochondrial function. Biological
624 Reproduction 72:562-567.
625 Gallach, M. and E. Betran. 2011. Intralocus sexual conflict resolved through gene
626 duplication. Trends in Ecology and Evolution 26:222-228.
627 Gallach, M., C. Chandrasekaran, and E. Betran. 2010. Analyses of nuclearly
628 encoded mitochondrial genes suggest gene duplication as a mechanism
629 for resolving intralocus sexually antagonistic conflict in Drosophila.
630 Genome Biology and Evolution 2:835-850.
631 Gemmell, N. J., V. J. Metcalf, and F. W. Allendorf. 2004. Mother's curse: the effect
632 of mtDNA on individual fitness and population viability. Trends in Ecology
633 and Evolution 19:238-244.
634 Gershoni, M., A. R. Templeton, and D. Mishmar. 2009. Mitochondrial
635 bioenergetics as a major motive force of speciation. Bioessays 31:642-
636 650.
637 Hayward, A. and J. F. Gillooly. 2011. The cost of sex: Quantifying energetic
638 investment in gamete production by males and females. PLoS ONE 6.
639 Hoekstra, L. A., M. A. Siddiq, and K. L. Montooth. 2013. Pleiotropic effects of a
640 mitochondrial-nuclear incompatibility depend upon the accelerating
641 effect of temperature in Drosophila. Genetics 195:1129-1139.
642 Holyoake, A. J., P. McHugh, M. Wu, S. O'Carroll, P. Benny, I. L. Sin, and F. Y. Sin.
643 2001. High incidence of single nucleotide substitutions in the

644 mitochondrial genome is associated with poor semen parameters in men.
645 International Journal of Andrology 24:175-182.
646 Immler, S., S. Pitnick, G. A. Parker, K. L. Durrant, S. Lupold, S. Calhim, and T. R.
647 Birkhead. 2011. Resolving variation in the reproductive tradeoff between
648 sperm size and number. Proc Natl Acad Sci U S A 108:5325-5330.
649 Innocenti, P., E. H. Morrow, and D. K. Dowling. 2011. Experimental evidence
650 supports a sex-specific selective sieve in mitochondrial genome evolution.
651 Science 332:845-848.
652 Kageyama, D., S. Narita, T. Imamura, and A. Miyanoshita. 2010. Detection and
653 identification of Wolbachia endosymbionts from laboratory stocks of
654 stored-product insect pests and their parasitoids. J Stored Prod Res
655 46:13-19.
656 Katsuki, M., Y. Toquenaga, and T. Miyatake. 2013. Larval competition causes the
657 difference in male ejaculate expenditure in *Callosobruchus maculatus*.
658 Popul Ecol 55:493-498.
659 Kazancioglu, E. and G. Arnqvist. 2013. The maintenance of mitochondrial genetic
660 variation by negative frequency-dependent selection. Ecol Lett 17:22-27.
661 Kleiber, M. 1961. The fire of life: An introduction to animal energetics. John Wiley
662 and Sons, Inc., New York.
663 Kondo, N., M. Shimada, and T. Fukatsu. 1999. High prevalence of Wolbachia in the
664 azuki bean beetle *Callosobruchus chinensis* (Coleoptera, Bruchidae).
665 Zoological Science 16:955-962.
666 Kotiaho, J. S. and L. W. Simmons. 2003. Longevity cost of reproduction for males
667 but no longevity cost of mating or courtship for females in the male-
668 dimorphic dung beetle *Onthophagus binodis*. J Insect Physiol 49:817-822.
669 Lemos, B., L. O. Araripe, and D. L. Hartl. 2008. Polymorphic Y chromosomes
670 harbor cryptic variation with manifold functional consequences. Science
671 319:91-93.
672 Levin, L., A. Blumberg, G. Barshad, and D. Mishmar. 2014. Mito-nuclear co-
673 evolution: the positive and negative sides of functional ancient mutations.
674 Frontiers in genetics 5:448.
675 Mappes, J., R. V. Alatalo, J. Kotiaho, and S. Parri. 1996. Viability costs of condition-
676 dependent sexual male display in a drumming wolf spider. P Roy Soc B-
677 Biol Sci 263:785-789.
678 Martin, O. Y. and D. J. Hosken. 2004. Copulation reduces male but not female
679 longevity in *Saltella sphondylli* (Diptera: Sepsidae). J Evol Biol 17:357-362.
680 Meiklejohn, C. D., K. L. Montooth, and D. M. Rand. 2007. Positive and negative
681 selection on the mitochondrial genome. Trends Genet 23:259-263.
682 Oliver, C. and C. Cordero. 2009. Multiple mating reduces male survivorship but
683 not ejaculate size in the polygamous insect *Stenomacra marginella*
684 (Heteroptera: Largidae). Evol Ecol 23:417-424.
685 Olsson, M., T. Madsen, and R. Shine. 1997. Is sperm really so cheap? Costs of
686 reproduction in male adders, *Vipera berus*. P Roy Soc B-Biol Sci 264:455-
687 459.
688 Oppliger, A., D. J. Hosken, and G. Ribi. 1998. Snail sperm production
689 characteristics vary with sperm competition risk. P Roy Soc B-Biol Sci
690 265:1527-1534.
691 Parker, G. A. 1979. Sexual selection and sexual conflict. Academic Press.

692 Paukku, S. and J. S. Kotiaho. 2005. Cost of reproduction in *Callosobruchus*
693 *maculatus*: effects of mating on male longevity and the effect of male
694 mating status on female longevity. J Insect Physiol 51:1220-1226.
695 Pitnick, S. 1996. Investment in testes and the cost of making long sperm in
696 Drosophila. Am Nat 148:57-80.
697 Preston, B. T., I. R. Stevenson, J. M. Pemberton, and K. Wilson. 2001. Dominant
698 rams lose out by sperm depletion - A waning success in siring counters a
699 ram's high score in competition for ewes. Nature 409:681-682.
700 Rajender, S., P. Rahul, and A. A. Mahdi. 2010. Mitochondria, spermatogenesis and
701 male infertility. Mitochondrion 10:419-428.
702 Ramalho-Santos, J., S. Varum, S. Amaral, P. C. Mota, A. P. Sousa, and A. Amaral.
703 2009. Mitochondrial functionality in reproduction: from gonads and
704 gametes to embryos and embryonic stem cells. Hum Reprod Update
705 15:553-572.
706 Rand, D. M., A. G. Clark, and L. Kann. 2001. Sexually antagonistic cytonuclear
707 fitness interactions in *Drosophila melanogaster*. Genetics 159:173-187.
708 Rand, D. M., R. A. Haney, and A. J. Fry. 2004. Cytonuclear coevolution: the
709 genomics of cooperation. Trends Ecol Evol 19:645-653.
710 RDevelopmentCoreTeam. 2011. R: a language and environment for statistical
711 computing, Vienna (Austria).
712 Rice, W. R. and S. D. Gaines. 1994. 'Heads I win, tails you lose': testing directional
713 alternative hypotheses in ecological and evolutionary research. Trends
714 Ecol Evol 9:235-237.
715 Rigaud, T. and J. Moreau. 2004. A cost of Wolbachia-induced sex reversal and
716 female-biased sex ratios: decrease in female fertility after sperm
717 depletion in a terrestrial isopod. P Roy Soc B-Biol Sci 271:1941-1946.
718 Rönn, J. L., M. Katvala, and G. Arnqvist. 2008. Interspecific variation in ejaculate
719 allocation and associated effects on female fitness in seed beetles. J Evol
720 Biol 21:461-470.
721 Sackton, T. B., H. Montenegro, D. L. Hartl, and B. Lemos. 2011. Interspecific Y
722 chromosome introgressions disrupt testis-specific gene expression and
723 male reproductive phenotypes in Drosophila. Proc Natl Acad Sci U S A
724 108:17046-17051.
725 Salehialavi, Y., K. Fritzsche, and G. Arnqvist. 2011. The cost of mating and mutual
726 mate choice in two role-reversed honey locust beetles. Behav Ecol
727 22:1104-1113.
728 Savalli, U. M. and C. W. Fox. 1999a. The effect of male mating history on paternal
729 investment, fecundity and female remating in the seed beetle
730 *Callosobruchus maculatus*. Funct Ecol.
731 Savalli, U. M. and C. W. Fox. 1999b. The effect of male size, age, and mating
732 behavior on sexual selection in the seed beetle *Callosobruchus maculatus*.
733 Ethol Ecol Evol 11:49-60.
734 Scharf, I., F. Peter, and O. Y. Martin. 2013. Reproductive trade-offs and direct
735 costs for males in arthropods. Evolutionary Biology 40:169-184.
736 Simmons, L. W. and J. S. Kotiaho. 2007. The effects of reproduction on courtship,
737 fertility and longevity within and between alternative male mating tactics
738 of the horned beetle, *Onthophagus binodis*. J Evol Biol 20:488-495.
739 Sirot, L. K., N. A. Buehner, A. C. Fiumera, and M. F. Wolfner. 2009. Seminal fluid
740 protein depletion and replenishment in the fruit fly, Drosophila

741 melanogaster: an ELISA-based method for tracking individual ejaculates.
742 Behavioral Ecology and Sociobiology 63:1505-1513.
743 Smith, S., C. Turbill, and F. Suchentrunk. 2010. Introducing mother's curse: low
744 male fertility associated with an imported mtDNA haplotype in a captive
745 colony of brown hares. Mol Ecol 19:36-43.
746 South, S. H., D. Steiner, and G. Arnqvist. 2009. Male mating costs in a polygynous
747 mosquito with ornaments expressed in both sexes. P Roy Soc B-Biol Sci
748 276:3671-3678.
749 Speakman, J. R. 2008. The physiological costs of reproduction in small mammals.
750 Philosophical Transactions of the Royal Society London B Biological
751 Sciences 363:375-398.
752 Takakura, K. 1999. Active female courtship behavior and male nutritional
753 contribution to female fecundity in *Bruchidius dorsalis* (*Fahraeus*)
754 (Coleoptera : Bruchidae). Researches on Population Ecology 41:269-273.
755 Thomsen, R., J. Soltis, M. Matsubara, K. Matsubayashi, M. Onuma, and O.
756 Takenaka. 2006. How costly are ejaculates for Japanese macaques?
757 Primates 47:272-274.
758 Trier, C. N., J. S. Hermansen, G. P. Saetre, and R. I. Bailey. 2014. Evidence for mito-
759 nuclear and sex-linked reproductive barriers between the hybrid italian
760 sparrow and its parent species. PLoS Genet 10:e1004075.
761 Trivers, R. L. 1972. Parental investment and sexual selection. Aldine Publishing
762 Company.
763 Tuda, M., J. Ronn, S. Buranapanichpan, N. Wasano, and G. Arnqvist. 2006.
764 Evolutionary diversification of the bean beetle genus *Callosobruchus*
765 (Coleoptera: Bruchidae): traits associated with stored-product pest
766 status. Mol Ecol 15:3541-3551.
767 Wade, M. J. 2014. Paradox of mother's curse and the maternally provisioned
768 offspring microbiome. Cold Spring Harbor perspectives in biology
769 6:a017541.
770 Wade, M. J. and Y. Brandvain. 2009. Reversing mother's curse: selection on male
771 mitochondrial fitness effects. Evolution 63:1084-1089.
772 Wade, M. J. and S. M. Shuster. 2003. Mating systems and strategies. Princeton
773 University Press, Princeton, New Jersey.
774 Wedell, N., M. J. G. Gage, and G. A. Parker. 2002. Sperm competition, male
775 prudence and sperm-limited females. Trends Ecol Evol 17:313-320.
776 Wightman, J. A. 1978. The ecology of *Callosobruchus analis* (Coleoptera:
777 Bruchidae): energetics and energy reserves of the adults. The Journal of
778 Animal Ecology:131-142.
779 Yee, W. K. W., K. L. Sutton, and D. K. Dowling. 2013. In vivo male fertility is
780 affected by naturally occurring mitochondrial haplotypes. Curr Biol
781 23:R55-R56.
782 Zhu, C. T., P. Ingelmo, and D. M. Rand. 2014. GxGxE for lifespan in *Drosophila*:
783 mitochondrial, nuclear, and dietary interactions that modify longevity.
784 PLoS Genet 10:e1004354.
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786

Tables

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	Df	*P*	X^2	Df	*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 X 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 X treatment	0.4	2	0.8182	0.2	2	0.8977
Nuclear X treatment	1.8	2	0.4031	0.3	2	0.8456
Mitochondrial X nuclear X 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		

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

Fixed effects	X^2	**Df**	***P***
Nuclear lineage	2.8	2	0.2470
Mitochondrial lineage	10.0	2	**0.0067**
Mitochondrial X 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 X 1st ejaculate	7.9	2	**0.0194**
Nuclear X 1st ejaculate	6.2	2	**0.0457**
Mitochondrial X nuclear X 1st ejaculate	22.7	4	**0.0001**
Mitochondrial X 1st copulation dur.	0.5	2	0.7599
Nuclear X 1st copulation dur.	9.7	2	**0.0077**
Mitochondrial X nuclear X 1st copulation dur.	2.6	4	0.6244
Random effects	**Variance *(σ^2)***		
Line	1.472×10^{-20}		
Residual	0.00009182		

798

799

800 **Table 3.** Mixed model of the effects of metabolic rate, mitochondrial, nuclear,
801 and mitonuclear genotype on variation in the weight of the second ejaculate. N =
802 74 male triads.

803

Fixed effects	X^2	**Df**	***P***
Nuclear lineage	35.1	2	**<0.0001**
Mitochondrial lineage	25.5	2	**<0.0001**
Mitochondrial X 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 X 1st ejaculate	8.04	2	**0.0180**
Mitochondrial X 1st ejaculate	1.33	2	0.5125
Mitochondrial X nuclear X 1st ejaculate	14.5	4	**0.0059**
Total CO_2	0.04	1	0.8363
Mitochondrial X CO_2	7.22	2	**0.0271**
Nuclear X CO_2	1.97	2	0.3735
Mitochondrial X nuclear X CO_2	2.33	4	0.6746
Random effects	**Variance *(σ^2)***		
Line	0.00		
Residual	8.886×10^{-9}		

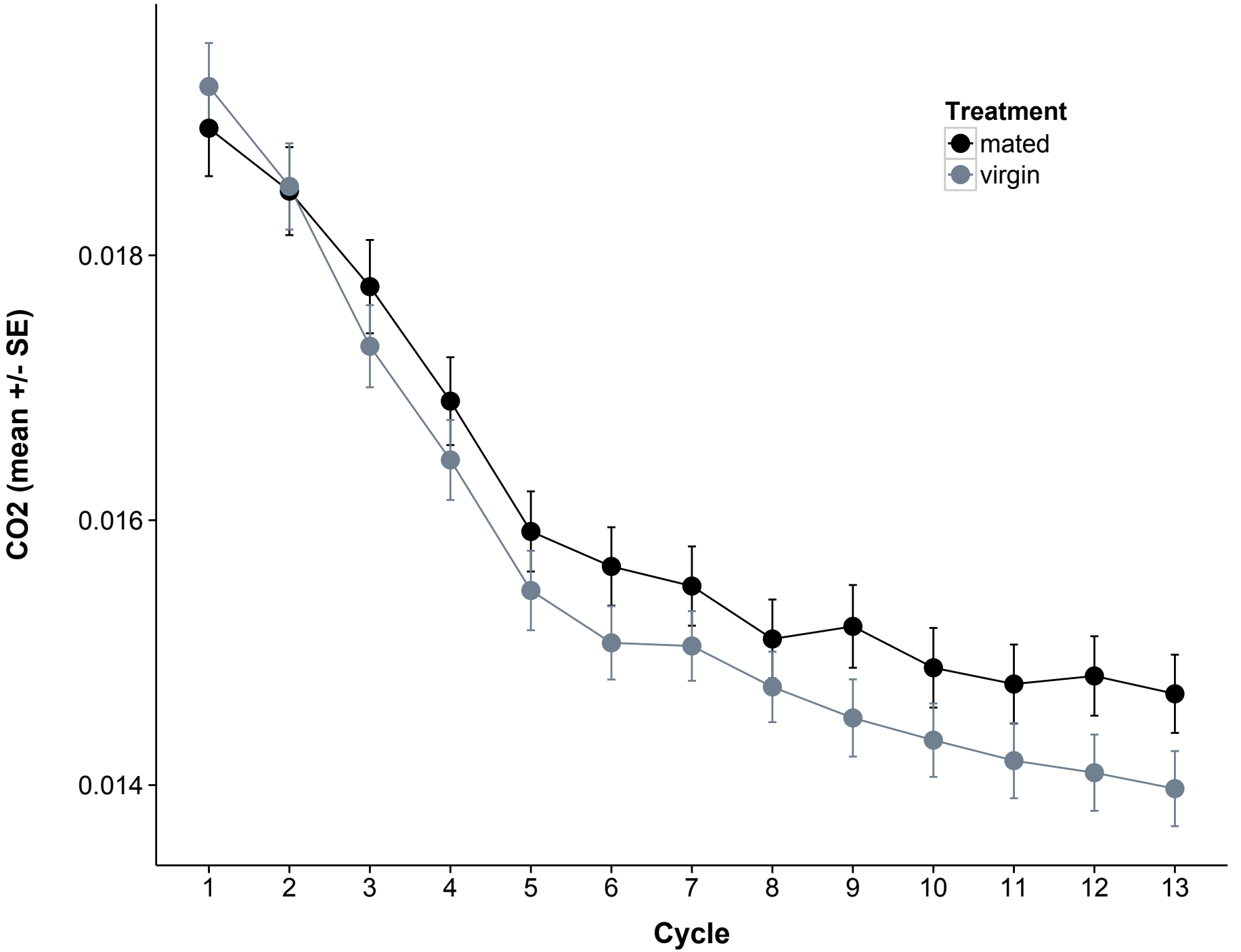
804

Figure Legends

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

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


Treatment
mated
virgin
CO2 (mean +/- SE)
0.018
0.016
0.014
1
2
3
4
5
6
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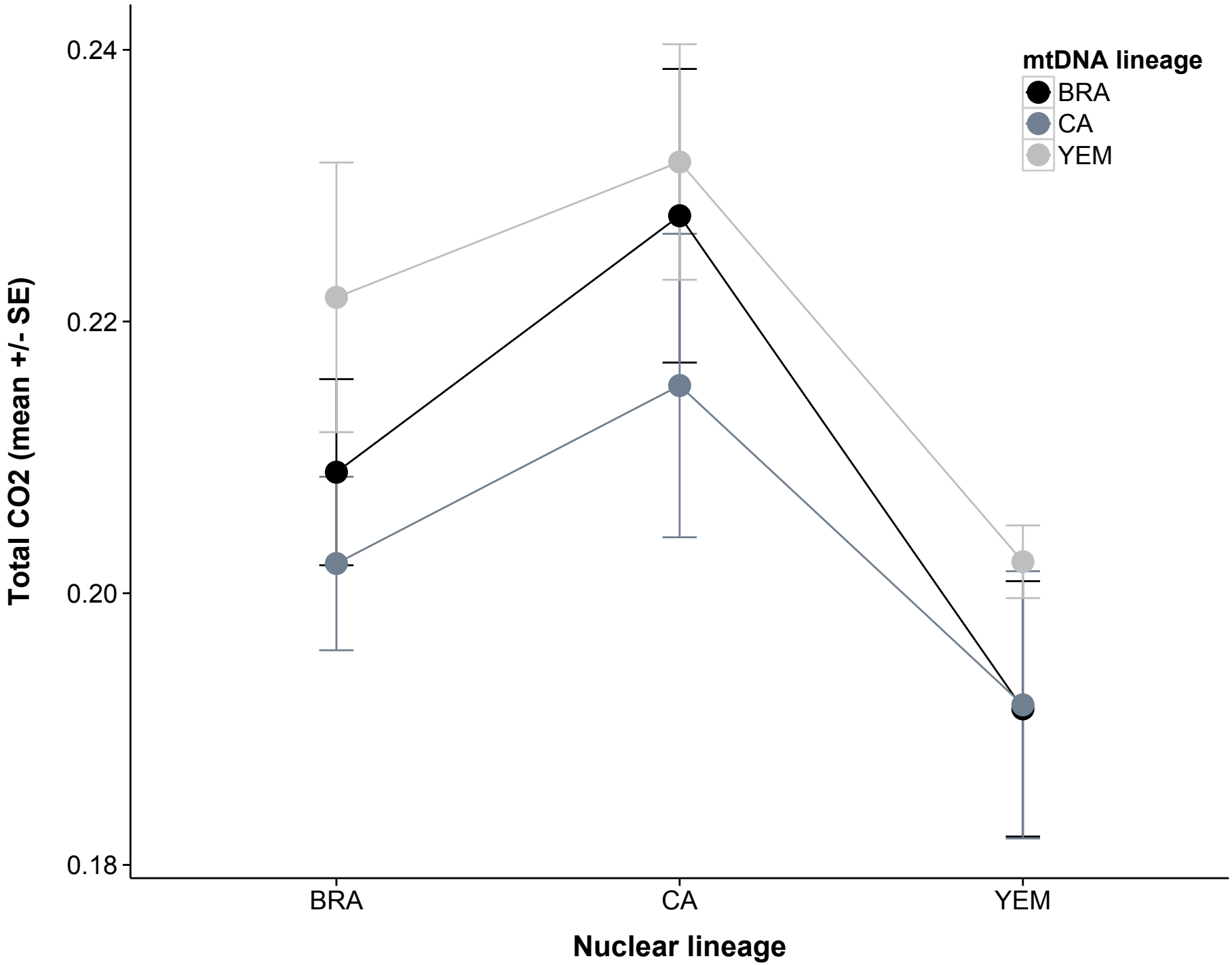
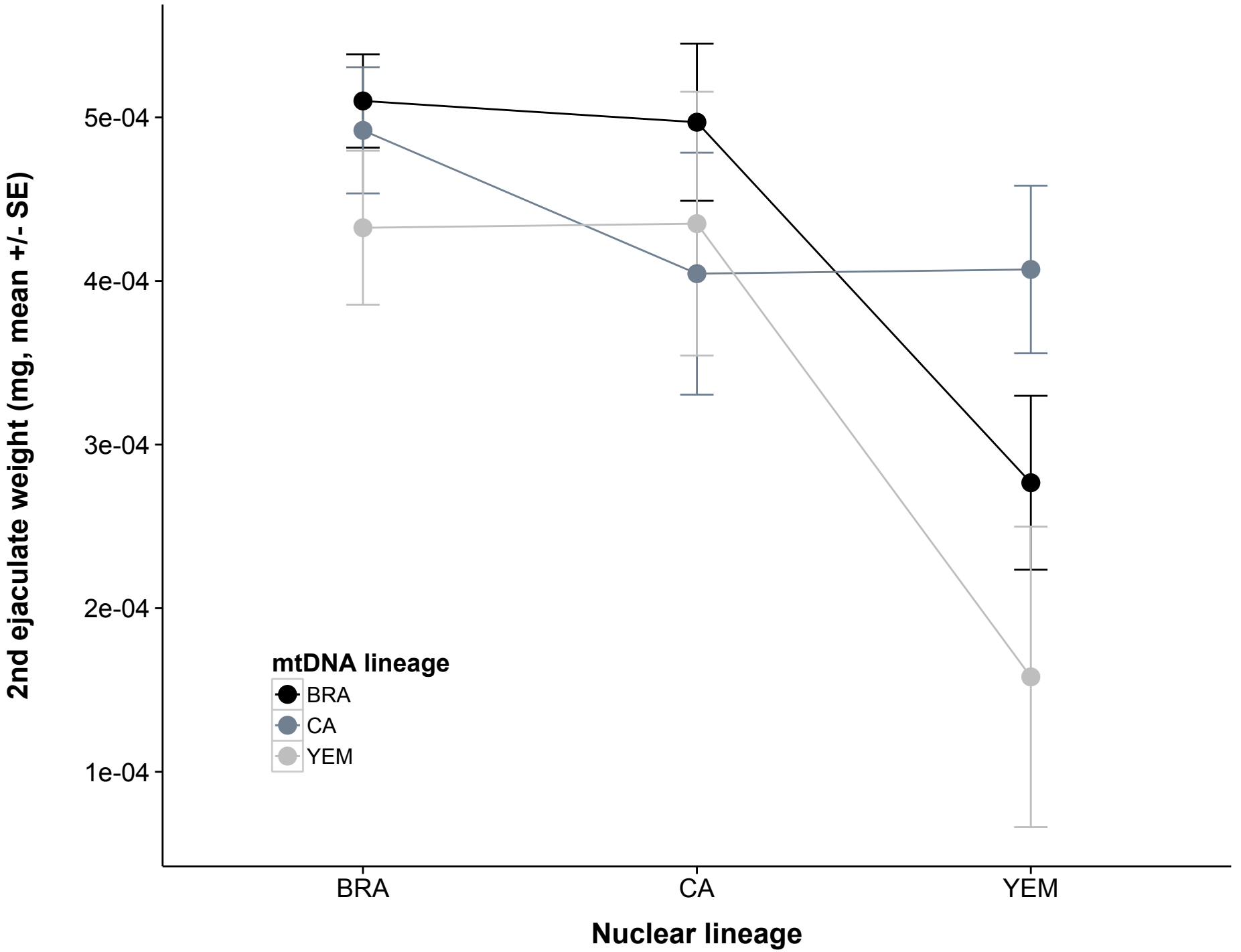
8
9
10
11
12
13
Cycle


mtDNA lineage
BRA
CA
YEM
0.24
0.22
0.20
0.18
Total CO2 (mean +/- SE)
BRA
CA
YEM
Nuclear lineage


2nd ejaculate weight (mg, mean +/- SE)
5e-04
4e-04
3e-04
2e-04
1e-04
mtDNA lineage
BRA
CA
YEM
BRA
CA
YEM
Nuclear lineage