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No evidence of mitochondrial genetic variation for sperm competition within a population of *Drosophila melanogaster*

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Abstract

Recent studies have advocated a role for mitochondrial DNA (mtDNA) in sperm competition. This is controversial because earlier theory and empirical work suggested that mitochondrial genetic variation for fitness is low. Yet, such studies dealt only with females and did not consider that variation that is neutral when expressed in females, might be non-neutral in males as, in most species, mtDNA is never selected in males. We measured male ability to compete for fertilizations, at young and late ages, across 25 cytoplasms expressed in three different nuclear genetic backgrounds, within a population of *Drosophila melanogaster*. We found no cytoplasmic (thus no mtDNA) genetic variation for either male offence or offensive sperm competitiveness. This contrasts with previous findings demonstrating cytoplasmic genetic variation for female fitness and female ageing across these same lines. Taken together, this suggests that mitochondrial genes do not contribute to variation in sperm competition at the within-population level.

Introduction

Sperm competition is thought to be a potent evolutionary force in many species, as a substantial component of a male's fitness should be determined by his ability to compete with the sperm of other males (Parker, 1970; Birkhead & Møller, 1998, Simmons, 2001). Most genetic studies of sperm competitive ability have concluded that the heritability of this trait is low (Gilchrist & Partridge, 1997; Hughes, 1997; Hosken *et al.*, 2001; Simmons, 2003; Friberg *et al.*, 2005; Konior *et al.*, 2005; but see Radwan, 2003). However, several recent studies have detected relatively high maternal heritabilities for traits related to sperm competitive ability (Ward, 2000; Morrow & Gage, 2001; Froman *et al.*, 2002; Simmons & Kotiaho, 2002; Simmons, 2003; Birkhead *et al.*, 2005). Some of these concluded that these heritabilities were attributable to X-linked variation for these traits (Ward, 2000; Morrow & Gage, 2001), whereas others suggested that mitochondrial-linked variation was likely to be important (Froman *et al.*, 2002; Simmons, 2003; Birkhead *et al.*, 2005).

Indeed, numerous authors have now suggested that the mitochondria may play an important role in sperm competition (e.g. Frank & Hurst, 1996; Pizzari & Birkhead, 2002; Pizzari *et al.*, 2002; Gemmell *et al.*, 2004; Arnqvist & Rowe, 2005; Zeh & Zeh, 2005; Dowling *et al.*, 2007c). A chief reason for this is that the competitive ability of sperm is assumed to be highly dependent on its motility, and as mitochondrial respiration is an important source of the fuel (ATP) necessarily for metabolic activity (hence sperm motility), mitochondrial function could play a vital role in sperm competition. Initial evidence for mitochondrial involvement in sperm competition was provided by studies that showed positive correlations between the volume of the sperm mid-piece, which contains the mitochondria, and the risk of sperm competition in shore birds (Johnson & Briskie, 1999), mammals (Anderson *et al.*, 2005; but see Gage & Freckleton, 2003 for contradicting results) and primates (Anderson & Dixson, 2002). Although these correlations suggest a link between mitochondria and sperm competition, it should be noted that such variation between individuals and species is probably explained by the number of mitochondria packed into a sperm, a trait that

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is presumably determined by nuclear genes. Nonetheless, other studies have found associations between mitochondrial DNA (mtDNA) variation (particularly in the form of point mutations or deletions) and sperm motility in humans (Kao *et al.*, 1998; Holyoake *et al.*, 2001) and birds (Froman & Kirby, 2005), and sperm motility is a known correlate of sperm competitive success, at least in birds (Birkhead *et al.*, 1999).

Furthermore, mitochondrial genetic variation could also influence sperm competitive ability through mechanisms other than the differential motility of sperm. For instance, given the high energetic demand required to produce vast numbers of viable sperm (e.g. Pitnick, 1996; Olsson *et al.*, 1997), sperm production *per se* should be dependent on uncompromised mitochondrial function. This suggestion is supported by a recent study that found that the viability and morphology of sperm, in a seed beetle, is tied to cytoplasmic (presumably mitochondrial) genetic variation (Dowling *et al.*, 2007c). Finally, we note that high levels of reactive oxygen species (ROS) in the ejaculate in humans have been linked to an increased number of sperm defects (Aziz *et al.*, 2004), and that the mitochondria are a primary source of ROS production (Harman, 1972), thus providing another potential mechanism by which mitochondrial genetic variation may affect the competitive ability of sperm.

Thus, from a conceptual standpoint, there appears to be ample scope for the mitochondria, and hence mitochondrial genetic variation, to play a role in influencing sperm competitive ability, and this is supported by the empirical studies that we reviewed above showing mitochondrial genetic variation for certain sperm traits (motility, viability and morphology). However, it is unclear whether the mitochondrial genetic variation underlying these sperm traits will translate into mitochondrial genetic variation underlying sperm competitive ability *per se*, a trait which is much more closely related to male fitness than the aforementioned sperm traits. On the one hand, we note that earlier genetic models of within-population mitochondrial genetic variation for fitness predicted that such variation will generally be low (e.g. Clark, 1984; Gregorius & Ross, 1984; Takahata, 1984; Clark & Lyckegaard, 1988). However, recent theoretical (Rand *et al.*, 2001) and empirical (Rand *et al.*, 2001; Dowling *et al.*, 2007b) advances have indicated that these conclusions may have been based on overly simplified assumptions. In addition to this, it should be noted that all of the previous genetic models of mitochondrial fitness variation have been formulated and applied specifically around females, given that the cytoplasm (and mtDNA) is maternally transmitted, and will thus only respond to selection in females (Birky, 2001). However, an intriguing quirk that arises from the strict maternal transmission of mtDNA is that it opens up the possibility for male expression-specific, non-neutral mitochondrial genetic variation to accumulate within a population, if this variation is selectively neutral (or nearly so) when expressed in females (Frank & Hurst, 1996). It is conceivable that such male expression-specific mtDNA variation might be particularly likely to accrue in male-limited traits, or in male homologues of sexually dimorphic traits (e.g. the seminal fluid and traits related to the sperm) where the demands on mitochondrial function may differ between the sexes.

In sum, there are thus several lines of evidence to indicate that mitochondrial variation may play an important role in sperm competition within populations, although no formal test of this idea has been conducted to date. Here, we test whether cytoplasmic genetic variation underlies male offensive (i.e. when mating with a nonvirgin female) ability in sperm competition, in both young and old males, within a population of *Drosophila melanogaster*. Our experimental design provides a powerful test for the presence of within-population cytoplasmic genetic variation for male traits in general, and for sperm competition in particular.

Methods

Fly stocks

Flies used in this experiment came from a large (~1792 individuals), outbred laboratory-adapted population of *D. melanogaster* (LH_M). This population is cultured on a 14-day discrete generation cycle. In addition to LH_M, we used a replica of this population, LH_M-*bw*. This population is homozygous for the recessive eye mutation *bw* that causes flies to have brown instead of red eyes. This population was created earlier, through sequential backcrossing of the *bw* allele into the LH_M genetic background over nine generations. Flies were maintained at 25 °C and 75% RH on a 12-h : 12-h light : dark cycle. For a detailed description of the LH_M population, see Rice *et al.* (2005). CO_2 anaesthesia was used when transferring flies throughout the experiment, unless otherwise noted.

Construction of cytoplasmic and inbred lines

As mitochondria and cytoplasms are co-transmitted, we created cytoplasmic lines (cyto-lines) by randomly sampling 25 mated females from the LH_M base population. Each of these females was used to found a separate cyto-line that was fixed for its cytoplasm. To disassociate the cytoplasm from the nuclear genetic background with which it was originally associated, daughters of each cyto-line were backcrossed with randomly selected males from the base population over 27 successive generations. At the end of this backcrossing protocol, approximately 99.92% of the original nuclear background had been replaced. All larvae were treated with tetracycline hydrochloride at generation 26 of the backcrossing protocol, to ensure they were not infected with any cytoplasmic bacteria (Hoffmann *et al.*, 1986, 1998).

Inbred lines were constructed by randomly collecting mated females from the LH_M population. Virgin offspring from these females were then brother–sister mated for nine consecutive generations. See Dowling *et al.* (2007b), for a full description of the construction of these same cyto- and inbred lines.

Experimental design

The experiment was designed to measure cytoplasmic genetic variation across the 25 cyto-lines for: (i) male offence (a joint measure of focal male ability to induce mated females to remate and the ability to displace already present sperm); and ii) offensive sperm competitive ability (focal male ability to displace sperm in mated females when remating) in young and old males, over three different haploid nuclear genetic backgrounds. The experiment was conducted in three independent blocks that were separated in time. In each block, 90 virgin females were collected, within 2–7 h of eclosing, from each of the 25 cyto-lines. These females were divided and stored by 10 across nine vials. At the same time, 750 males (0–1 day old) were collected from each of the three inbred lines and also stored in groups of 10 per vial. Two days later, the inbred males were introduced to the virgin females from the cyto-lines, in all possible cyto-line × inbred line combinations (see Fig. 1), with each combination replicated over three vials. The flies mated and resided in these vials for 24 h, after which they were discarded and the number of eggs trimmed to 150–200 per vial. Upon eclosion 9 days later, 18 male flies were collected over the three vials for each cyto-line × inbred line combination, thus 'cyto-nuclear genotype', and stored in one vial per cyto-nuclear type, to be assayed for male offence traits (see above) when 3 days old (young age class). At the same time, 60 additional males (stored by 20 across three vials) were collected to be assayed for the same male offence traits when 23 days old (old age class). All of the focal males, of both age classes, had inherited their cytoplasmic genotype along with a random haploid nuclear genome from their mothers, whereas the second haploid genome, inherited from their fathers, came from one of the inbred lines and was thus controlled (see Fig. 1). Thus, we generated focal males of 75 distinct cyto-nuclear genotypes, to be assayed at two age classes, per block.

Both the competitor males, which the focal males would compete with, and the females, which the males were to compete over, were derived from LH_M-*bw*. The LH_M-*bw* males were collected and stored in vials of 30 and virgin LH_M-*bw* females were collected and stored in vials of 20. As the focus of this study was the competitive ability of the focal males from an offensive point of view, the LH_M-*bw* females were first mated to the LH_M-*bw* competitor males. Specifically, when 3 days old, the LH_M-*bw* males of a given vial were introduced to the LH_M-*bw* females (2 days old) of a corresponding vial (without the use of anaesthesia) for 2 h. Virtually all virgin females of this age, housed with a 50% excess of males, mated once and only once in this population, during this time period (Holland & Rice, 1999; Rice, 1996). After the 2-h mating period, the LH_M-*bw* males were discarded and 16 of the 20 LH_M-*bw* females were transferred to a fresh vial together with 18 males from one of the focal cyto-nuclear male genotypes. In these adult competition vials, the females competed for a limited resource (live yeast, 11.6 mg dry weight), whereas the focal males had the opportunity to mate with the females and displace sperm from the previous mating.

Two days later, the males and females of each vial were separated and each female was placed in an individual

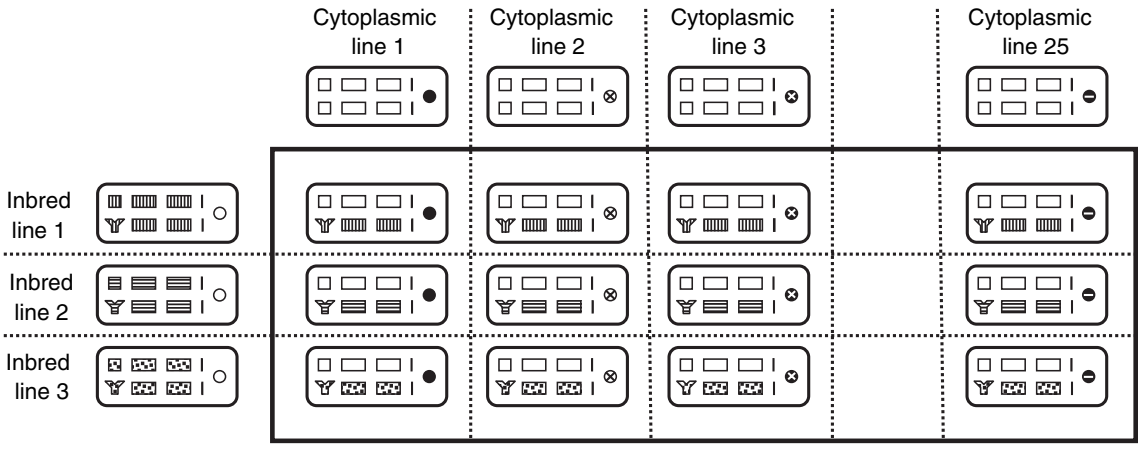

Fig. 1 Females of the 25 cyto-lines were crossed with males from three inbred lines in a full factorial design. Bars denote chromosomes, circles denote cytoplasms and Ys denote Y-chromosomes. The shading of the chromosomes and cytoplasms indicates their origin. White bars indicate random chromosomes from the base population and patterned bars indicate chromosomes derived from one of the inbred lines.

vial together with one of the males from the same remating vial (to mimic the standard rearing conditions in which females are exposed to males during ovipositioning). We note that rematings were not controlled and females could potentially have remated several times. However, this failure to control for remating rates is unlikely to have obscured our interpretation of the results, and the reasons for this are explained within the Discussion section. An incision was made in the medium of each vial to further entice females to oviposit. The pairs then spent 18 h in these vials before they were discarded. This protocol mimics the rearing conditions of the base population, in terms of the average amount of live yeast consumed by females, the timing of matings and the timing and length of the oviposition period. Ten days later, offspring emerging from each vial were counted and their eye colour scored. As the females and the competitor males were both homozygous for the recessive mutation *bw*, offspring fathered by the competitor males had brown eyes, whereas offspring fathered by the focal males were heterozygous for the recessive mutation and thus had red eyes. All vials were rechecked on day 11 and all flies hatching up to this point were included in our measures, which is consistent with the normal culturing protocol of this population.

The additional 60 focal males per cyto-nuclear genotype that were collected (old age class) were transferred, without CO_2 anaesthesia, to fresh vials every fourth to seventh day (in unison across lines and blocks) until they were 23 days old. From this point, they were assayed in exactly the same way as for the males of the young age class. A surplus amount of males were initially collected for this component of the experiment to ensure that enough males were still alive per cyto-nuclear genotype at 23 days of age to complete the assay. However, in retrospect, most of the 60 males per cyto-nuclear genotype survived this 23-day period.

Overall, approximately 96 broods were scored for each of the 75 cyto-nuclear genotypes over three blocks. A few females did not produce any offspring and were subsequently removed from the analyses reported below. Data were accidentally lost from two cyto-line × nuclear combinations during block 2 and one cyto-line × nuclear combination in block 3. In addition, one vial with 16 females was excluded from the analyses on the basis that, in total, they produced only one brown-eyed offspring. It is likely that the initial matings to the competitor males were unsuccessful in this particular vial, as this value was an order of magnitude less than that of the vial that produced the second least number of brown-eyed offspring, of 447 such vials. In total, 241 139 offspring were included in the analyses. The removal of the above females, and vial, from the analyses did not alter our ability to reject the null hypotheses, and all the results were qualitatively similar, regardless of whether these females and vial were included or not.

Statistical analysis

During the assay, 18 males from each specific cyto-line × nuclear × age class × block combination were given the opportunity to coerce 16 mated females to remate within a shared vial. Even though females were later separated into individual vials for ovipositioning, this created interdependencies between the females of a given remating vial as they potentially experienced a vial-specific environment during the assay. Thus, to create independent replicates, we used vial mean values when analysing the male offence traits. The level of replication per block and male age was therefore one (based on the mean of 16 or fewer females) for each cyto-nuclear genotype.

We scored two male offence-related traits; male offence and offensive sperm competitiveness: (1) male offence was defined as the mean number of red-eyed offspring produced across all 16 females per cyto-line × nuclear × age class × block, regardless of whether these females actually remated with the focal males or not. Thus, this measure encompasses both the ability of each focal male genotype to coerce females into remating, and, once remated, the ability to compete for fertilizations with sperm that is already present. (2) The measure of offensive sperm competitive ability aimed to extract only the sperm displacement component of the previous measure and therefore only included those females that produced at least one offspring from the focal males (i.e. one red-eyed offspring), so that only females that had mated with both focal and competitor males were included. Again, the mean number of red-eyed offspring produced by such females was used as the dependent variable.

Cyto-line, nuclear genetic background and block were all treated as random factors, whereas age was treated as a fixed factor. Interactions between random and fixed factors were all treated as random factors. No significant interactions involving the block effect were detected in any of these models, and, thus, for simplicity of presentation, we dropped such interactions from the final models. We note that dropping these interactions did not qualitatively or quantitatively alter the outcome of the analyses.

Variance components, and their 95% CIs, were estimated using the restricted maximum likelihood (REML) algorithm (PROC MIXED statement) in SAS version 9.1 (SAS Institute Inc., Cary, NC, USA) and the Satterthwaite method was used to estimate degrees of freedom. In some cases, one or two females were lost during the assay and thus some replicates were based on the mean of fewer than 16 females. Furthermore, as described above, the estimate of male offensive sperm competitive ability was based only on females that actually remated and produced offspring from their second mating (i.e. often fewer than 16 females per vial). To compensate, all models were weighted by the number of females contributing to each replicate. Below, we present estimates from REML models having permitted the estimation of negative variance

components as such models yield unbiased estimates of the variance, whereas models in which variance components are bounded to zero (not permitted to go below zero) introduce a positive bias (B. Walsh, personal communication). The residuals from these models did not significantly depart from normality (Shapiro–Wilk's *W*-tests, all $P > 0.28$).

Our rationale for utilizing the number of offspring sired, rather than proportion of focal offspring, as the response variable in the analyses was twofold. First, our aim here was to estimate variance components for the measured traits on a relevant untransformed scale, which is problematic when using proportional data. We acknowledge that proportions are the traditional metric used to measure sperm competitive ability, and that this metric has its merits when comparing patterns of sperm competition across populations and species with different fecundities. Nonetheless, it is questionable whether this is the appropriate metric to use when measuring sperm competitive ability in a fitness context. Male relative fitness is determined by the number of offspring sired in relation to the average number of offspring sired by males in the population. That is, selection does not act directly on the proportion of offspring that a male sires when mating to a particular female, but rather the number of offspring, as this is what adds to his total count of sired offspring. In this regard, it is worth noting that a previous study of the same *Drosophila* population as used here has shown that male offensive sperm competitive ability is negatively related to female fitness (Friberg *et al.*, 2005), which indicates that proportion and number of offspring are not linearly related, adding support for the use of offspring numbers rather than proportions when measuring sperm competitive ability in a fitness context. Nonetheless, we note that analysing our data using a transformed proportional scale did not change the interpretation of our results.

Results

The variance component estimate for the factor cyto-line was not significantly different from zero in the models of male offence (Table 1 and Fig. 2a) and sperm competitive ability (Table 1 and Fig. 2b). Likewise, the variances for the interactions of key interest, cyto-line × background and cyto-line × age, were not significant (Table 1). Male age had no effect on either of the male offence traits (Table 1).

Table 1 Models of male offence and offensive sperm competitiveness.

Source	Variance estimate	SE	*Z*	*P*	Upper 95% CI	% Total variation
Male offence						
Cyto-line	−0.0499	0.3464	−0.14	0.8854	0.6290	−0.013
Background	2.8983	3.0903	0.94	0.3483	8.9552	0.744
Block	0.5195	0.6904	0.75	0.4517	1.8726	0.133
Cyto-line × background	0.8913	0.6138	1.45	0.1465	2.0943	0.229
Cyto-line × age	0.0477	0.3915	0.12	0.9030	0.8151	0.012
Background × age	0.1742	0.3082	0.57	0.5718	0.7782	0.045
Cyto-line × background × age	−5.2793	0.9800	−5.39	–	−3.3585	−1.356
Residual	390.22	32.3622	12.06	< 0.0001	462.31	100.205
Total	389.42					
Effect	Num. d.f., den. d.f.	*F*	*P*			
Age	1, 2	0.48	0.56			
Source	Variance estimate	SE	*Z*	*P*	Upper 95% CI	% Total variation
Offensive sperm competitiveness						
Cyto-line	0.0656	0.3338	0.20	0.8434	0.7202	0.019
Background	2.4765	2.5079	0.99	0.3234	7.3919	0.696
Block	0.5062	0.6661	0.76	0.4474	1.8119	0.142
Cyto-line × background	0.5776	0.5327	1.08	0.2782	1.6216	0.162
Cyto-line × age	0.1045	0.3849	0.27	0.7860	0.8590	0.029
Background × age	−0.1081	0.0320	−3.49	–	−0.0472	−0.030
Cyto-line × background × age	−4.9128	0.9252	−5.31	–	−3.0994	−1.382
Residual	356.9155	30.0145	11.89	< 0.0001	423.8838	100.363
Total	355.6085					
Effect	Num. d.f., den. d.f.	*F*	*P*			
Age	1, 1	59.15	0.0823			

Cyto-line, nuclear genetic background (background) and block were all modelled as random effects and age as a fixed effect. All interactions were modelled as random effects. Num. d.f. and den. d.f. are numerator and denominator degrees of freedom, respectively.

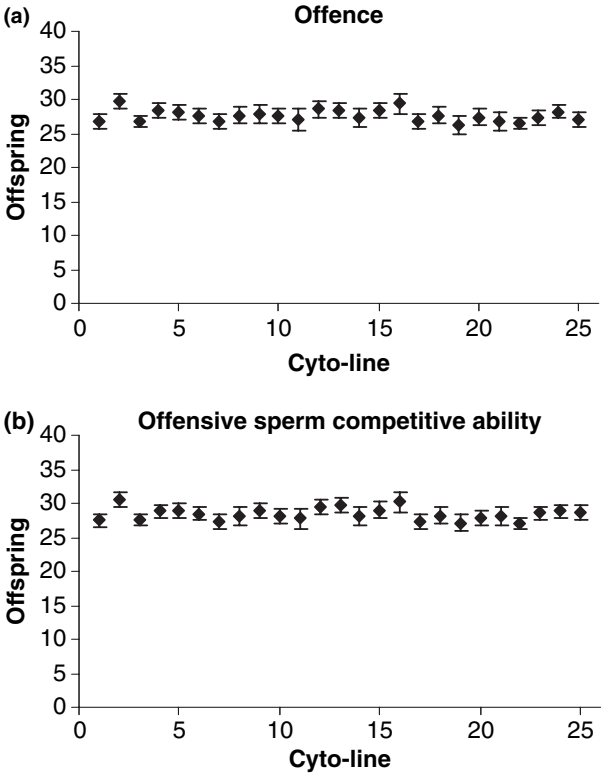

Fig. 2 Male offence (a) and offensive sperm competitive ability (b) for the 25 cyto-lines. Mean values and standard errors of the raw data are presented.

To put these nonsignificant results in perspective, and to evaluate the strength of our analyses, we estimated the 97.5% upper bounds of the percent the genetic factors and interactions in our models explained of the total phenotypic variation. To calculate these upper bounds, we divided the upper 95% confidence intervals of the estimated variances with the total phenotypic variation, both reported in Table 1, and multiplied this figure by 100. The total phenotypic variation reported in the models equals the total phenotypic variation in the population, despite the fact that we used mean values rather than individual observations as the level of statistical replication, as all mean values were weighted by the number of females contributing in the model. These upper bounds were all very small (all < 1%), indicating that if there is a biologically relevant cytoplasmic genetic effect on male offence or offensive sperm competitive ability, then this effect is extremely small (Table 2).

Table 2 The 97.5% upper bound of the per cent of the total phenotypic variation explained by the genetic components measured in Table 1.

	Offence	OSCA
Cyto-line	0.16	0.20
Background	2.30	2.08
Cyto-line × background	0.54	0.46
Cyto-line × age	0.21	0.24
Background × age	0.20	−0.01
Cyto-line × background × age	−0.86	−0.87

OSCA, offensive sperm competitive ability.

Discussion

We found no cytoplasmic, and hence no mitochondrial, genetic variation for male offence or male offensive sperm competitive ability, either as additive effects or as interactive effects with male age or the nuclear genetic background. These results stand in sharp contrast to the findings of two other studies that utilized the same cyto-lines and revealed cytoplasmic genetic effects on phenotypic variation in female fitness (via cyto-nuclear interactions) (Dowling *et al.*, 2007b) and female lifespan and ageing (additive cytoplasmic effects) (Maklakov *et al.*, 2006). Given these earlier results, and combined with the knowledge that cytoplasmic genetic variation is predicted to be more prevalent in males than in females and particularly so for highly sexually dimorphic and sex-limited traits, it must be asked why we found no cytoplasmic genetic variation for the male traits studied here.

The lack of a cytoplasmic/mitochondrial effect is unlikely to result from low statistical power. First, the experimental design employed was similar to the design of Dowling *et al.* (2007b), who used the same combination of 25 cyto-lines and three nuclear backgrounds, to detect cytoplasmic fitness effects. The difference between the two studies is that here we investigated male fitness components rather than female fitness and, further, that we examined the male fitness components at two age classes. Second, we actually scored about $2\frac{1}{2}\times$ more focal individuals in this experiment than Dowling *et al.* (2007b) did in their study in which they found sizable cytoplasmic × nuclear genetic effects. Third, the estimated nonsignificant genetic variation for cyto-line, cyto-line × background and cyto-line × age for both male traits was very small and, with 97.5% certainty, never exceeded 1% of the total phenotypic variation.

A potential explanation for the low levels of cytoplasmic variation might be that there is a lack of underlying molecular variation among the cyto-lines. In Dowling *et al.* (2007b), 2752 base pairs (about 14% of the total mtDNA in *D. melanogaster*) of mtDNA were sequenced per cyto-line. In that sample of the mitochondrial genome, only one base pair polymorphism was found that corresponded to an amino acid replacement from cytosine to tyrosine or *vice versa*. At first, this may seem a trivial amount of molecular variation and indeed this sequence variation could not be significantly tied to the complex nonadditive genetic variation found for female fitness (Dowling *et al.*, 2007b). However, the additive genetic variation found for female lifespan and rate

of ageing in (Maklakov *et al.*, 2006) was correlated with this mtDNA sequence variation (D.K. Dowling, A.A. Maklakov, U. Friberg and F. Hailer, unpublished data). This either indicates that this limited sequence variation is sufficient to produce substantial phenotypic effects or, alternatively, that further correlated sequence variation exists (in the mtDNA regions that we did not sequence) that caused these phenotypic effects. We suggest, on the basis of the findings of cytoplasmic genetic variation for female traits (Maklakov *et al.*, 2006; Dowling *et al.*, 2007b), that there was sufficient molecular variation in mtDNA present among the cyto-lines to enable the detection of any biologically relevant mtDNA effects on the male traits measured here.

A recent theoretical and empirical study demonstrated that cyto-nuclear fitness interactions are more likely to occur when the nuclear genes involved are X-linked and when selection on these interactions differs in the two sexes (Rand *et al.*, 2001). Although the cyto-nuclear interactions detected for female fitness in Dowling *et al.* (2007b) potentially could have involved X-linked genes (the 25 cyto-types were expressed in three controlled nuclear backgrounds consisting of autosomes II–IV and the X-chromosome), no such interactions were possible in this study (the nuclear background consisted of autosomes II–IV and the Y-chromosome, see Fig. 1). Our failure to test for cyto-X-chromosome interactions in this study is, thus, one possible explanation for the discrepancy between the results found here and those in Dowling *et al.* (2007b).

Finally, there is also the possibility that the associations between mitochondrial function and sperm motility, and sperm motility and sperm competitive ability found in birds and primates, for some reason do not carry over to insects. In mice, it has been found that glycolysis provides a nontrivial amount of the ATP used by the sperm (Mukai & Okuno, 2004), and perhaps this alternative pathway to ATP production is even more important in insects. However, as stated in the Introduction, there are two other plausible pathways (through differential sperm/ejaculate production or ROS production) via which mitochondrial genetic variation could have an effect on sperm competitiveness.

We also failed to find any additive nuclear genetic variation for sperm competitive ability in this study, despite the knowledge that such variation exists in this population (Friberg *et al.*, 2005). We can think of two possible explanations for this observation. Friberg *et al.* (2005) employed 35 nuclear genotypes, whereas we employed only three (as the focus of this study was on cytoplasmic genetic variation). One possibility is that the three haploid genetic backgrounds tested here, by chance, were relatively similar. This is consistent with the results of Dowling *et al.* (2007b), who also found no additive differences in female fitness between these same genetic backgrounds. An alternative explanation is that the backgrounds used here did not contribute an X-chromosome to the focal males, as described above, unlike the haploid genomes utilized in Friberg *et al.* (2005) (which consisted of a copy of an X-chromosome, autosome II–III but excluding autosome IV). This alternative is appealing given that two previous studies that found maternal heritability of traits involved in sperm competition concluded that variation in these traits was likely to be X-linked (Ward, 2000; Morrow & Gage, 2001).

In sum, these findings might suggest that the sperm traits found to have maternal heritabilities in earlier studies (see Introduction) are perhaps more likely to be X linked rather than mitochondrial linked. A lack of cytoplasmic involvement in sperm competition is also supported by a recent study, at the between-population level, which failed to find any cytoplasmic-encoded variation for this trait among five populations of the seed beetle, *Callosobruchus maculatus* (Dowling *et al.*, 2007a). On the other hand, we point out that certain studies have tied variation in sperm traits to the cytoplasm. For instance, Ruiz-Pesini *et al.* (1998, 2000) have shown that certain point mutations in mtDNA are associated with reduced sperm motility in humans and Dowling *et al.* (2007c) demonstrated experimentally that phenotypic variation in sperm length and sperm viability, in *C. maculatus*, is partly encoded by cytoplasmic, and presumably mitochondrial, genes.

Potential confounding factors

We note that several factors may have potentially confounded our test of cytoplasmic genetic variation for the male offence traits measured here. These are, however, unlikely to have affected the conclusions of the study for the reasons outlined below. First, given that rematings were not controlled in these experiments, females could potentially have remated several times during their 2 days in confinement with the focal males, thus confounding sperm competitiveness with remating rate in our measure of sperm competitive ability. However, this failure to control for multiple remating is unlikely to have been important for two reasons, both of which are based on the assumption that, for any given genotype, the binary remating rate (proportion of females that mate at least twice) will covary with the multiple remating rate (proportion of females that mate more than twice). First, Friberg *et al.* (2005) utilized a protocol identical to this one and found that the estimated standing nuclear additive genetic variance for offensive sperm competitive ability did not change appreciably when the potentially confounding effect of binary remating rate was statistically removed. This suggests that even if our estimates were inflated by multiple remating here, this would not confound our estimates of genetic variation in offensive sperm competitiveness. Second, the binary remating rate in the present experiment did not vary among male cytoplasmic ($Z = 0.0$, $P = 1.00$, total variance explained = 0%),

nuclear ($Z = 0.51$, $P = 0.61$, variance = 0%) or joint cyto-nuclear ($Z = 1.23$, $P = 0.22$, variance = 0.35%) genotypes. This finding suggests that if values of sperm competitive ability were inflated due to multiple remating, then this was consistent across the range of cytoplasmic, nuclear and joint cyto-nuclear genotypes.

Another potential criticism stemming from the remating assay is our failure to observe individual matings. Consequently, we were unable to distinguish between females that mated solely with either the first or second set of males and those females that mated with both sets of males but produced offspring from only one of these males (i.e. complete first or second male sperm precedence). We may thus have erroneously included some females in our measures of male offensive sperm competitive ability and male offence that had actually failed to mate with the first set of males and, likewise, erroneously excluded some females in our measure of male sperm competitive ability that mated with both sets of males but only produced offspring with the first set of males. However, we point out that the chief motivation for employing the protocol of mass matings used here was to greatly increase the statistical power of the experiment, as it enabled us to assay many more males than would otherwise have been feasible. Moreover, the number of females that were erroneously included and excluded would have been very small and thus unlikely to have affected the results. For instance, results from previous studies on the same population reveal that only five (1.8%) of 277 (Friberg, 2006) and 50 (1.1%) of 4420 (Dowling *et al.*, 2007b) virgin females failed to produce offspring after a 2-h mating period (with no subsequent exposure to a second set of males). At least some of these females would have failed to lay eggs because they (or their mates) were infertile and thus very few females would have actually failed to mate in the first instance. Furthermore, there cannot have been an association between the number of females that failed to mate in the first place and any of the male lines assayed here, as all males and females involved in the first matings were drawn randomly from the LH_M-*bw* population.

There may have also been a few cases in which females mated with both sets of males but only produced offspring from the first set, in which case they were misclassified as females that did not remate and thus erroneously removed from the sperm competitive ability analysis. However, we note that only 2.1% of all females failed to produce any offspring from the second males, suggesting that any misclassifications here would not have significant ramifications on our results. Second, given that P2 (the proportion of offspring sired by the second male when females mate twice) is very high in *D. melanogaster* (81% in this study averaged over the two age classes as estimated by our variable offensive sperm competitive ability), it seems unlikely that many of the females that only produced offspring from the first mating actually mated a second time. Nonetheless, we acknowledge that a few of these females were potentially misclassified as not having remated when they actually had, but point out that any of the misclassifications discussed above will only add minor and stochastic noise to the analyses and that any statistical power lost as a result of such noise was greatly offset by the increased power gained by employing the protocol of mass mating.

Our measures of male offence traits will also be partially confounded if egg-to-adult viability rates vary among the cyto-nuclear genotypes (Gilchrist & Partridge, 1997). Again, such a scenario is unlikely for the following two reasons. First, the performance of each cyto-nuclear genotype was assessed in an outbred nuclear background (i.e. the heterozygous state) and therefore genetic variation in viability will be small and unrelated to male fitness in the studied population (Chippindale *et al.*, 2001). Second, no correlation was found between P2 values and egg-to-adult viability across controlled haploid nuclear genotypes (hemiclones) in a previous study using this same population (Friberg *et al.*, 2005).

Conclusion

We tested for within-population cytoplasmic genetic variation in males for male offence traits at two age classes. Given that these traits are dependent on the function of seminal fluids (a sex-limited trait) and the male gamete (a highly sexually dimorphic trait), they are predicted to be especially sensitive to express such variation. Notably, we did not detect any variation in these traits that could be linked to the cytoplasm, and thus to mtDNA. Furthermore, these null results were associated with high statistical power, as we could conclude with 97.5% certainty that any putative cytoplasmic effect on these traits contributes to less than 1% of the total phenotypic variation. This result is consistent with the findings of Dowling *et al.* (2007a), who also failed to detect any cytoplasmic genetic effects on sperm competitiveness in *C. maculatus*, at the between-population level. If the estimated magnitude of the cytoplasmic contribution to sperm competitive ability for the population studied here proves to be general across taxa, then this will dispel the notion that mitochondrial genes play a profound role in offensive sperm competition.

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