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Effects of cytoplasmic genes on sperm viability and sperm morphology in a seed beetle: implications for sperm competition theory?

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Abstract

Sperm competition theory predicts that sperm traits influencing male fertilizing ability will evolve adaptively. However, it has been suggested that some sperm traits may be at least partly encoded by mitochondrial genes. If true, this may constrain the adaptive evolution of such traits because mitochondrial DNA (mtDNA) is maternally inherited and there is thus no selection on mtDNA in males. Phenotypic variation in such traits may nevertheless be high because mutations in mtDNA that have deleterious effects on male traits, but neutral or beneficial effects in females, may be maintained by random processes or selection in females. We used backcrossing to create introgression lines of seed beetles (*Callosobruchus maculatus*), carrying orthogonal combinations of distinct lineages of cytoplasmic and nuclear genes, and then assayed sperm viability and sperm length in all lines. We found sizeable cytoplasmic effects on both sperm traits and our analyses also suggested that the cytoplasmic effects varied across nuclear genetic backgrounds. We discuss some potential implications of these findings for sperm competition theory.

Introduction

The mitochondrial genome is generally regarded as a passive bystander in adaptive evolution (Rand, 2001). However, recent empirical advances have demonstrated that this assumption may not be valid (reviewed in Ballard & Whitlock, 2004; Ballard & Rand, 2005). For instance, it has been demonstrated that cytoplasmic (probably mitochondrial) genes are involved in epistatic interactions with nuclear genes, and that these interactions influence genetic variation in fitness components (e.g. Rand *et al.*, 2001; James & Ballard, 2003; Zeyl *et al.*, 2005). Further, recent theory suggests that such interactions may maintain within-population genetic polymorphism in mitochondrial/cytoplasmic genes (Rand *et al.*, 2001).

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Mitochondrial DNA (mtDNA) is maternally inherited in most anisogamous species and selection on mtDNA thus operates only in females. Males are effectively a 'dead end' for cytoplasmic genes (cf. Pizzari & Birkhead, 2002; Zeh, 2004). One intriguing consequence of this fact is that mutations accruing in the mitochondrial genome that have deleterious effects on male fitness but neutral, or even slightly deleterious, effects on female fitness can theoretically be maintained in a population (Frank & Hurst, 1996). If beneficial for females, selection will act to maintain such mutations despite the deleterious effects in males (Chippindale *et al.*, 2001; Rand *et al.*, 2001; Arnqvist & Rowe, 2002, 2005).

Sperm quality and fertilizing efficiency represent male traits that may potentially be compromised by mtDNA mutations that are harmful when expressed in males but not in females. One reason for this is that the sperm flagellum contains mitochondria that are potentially used to power sperm motility in both vertebrates (Mitchell *et al.*, 1976) and insects, even though in the latter the mitochondria fuse within the sperm to form two mitochondrial derivatives (Bigliardi *et al.*, 1970; Báo *et al.*,

1992, but see Baccetti *et al.*, 1973; Perotti, 1973; Werner *et al.*, 1999). Mutations may accrue in mtDNA that result in reduced sperm motility and/or viability, without impairing the somatic cellular function in either sex. This may be largely due to a distinct coupling between mtDNA and phenotypic function in sperm (Frank & Hurst, 1996). Alternatively, considering the potentially high energetic investment required to produce large numbers of functional sperm (Dewsbury, 1982; Van Voorhies, 1992; Pitnick & Markow, 1994; Pitnick, 1996; Olsson *et al.*, 1997) and the pivotal role of mitochondrial genes in energy production, polymorphism in mtDNA may result in the differential ability of different haplotypes to produce large numbers of viable, high-quality sperm.

Increased attention has been devoted to the possible role of mitochondrial genes in determining male sperm quality, particularly motility (see Moore & Reijo-Pera, 2000; Gemmell & Allendorf, 2001; Pizzari & Birkhead, 2002; Gemmell *et al.*, 2004). Some studies have identified associations between mitochondrial mutations and decreases in human sperm motility (Kao *et al.*, 1998; Ruiz-Pesini *et al.*, 2000; Holyoake *et al.*, 2001), while others have failed to do so (Cummins *et al.*, 1998; St John *et al.*, 2001). Such studies typically examined associations between known mtDNA deletions and sperm functioning in groups of humans with known fertility problems (e.g. Kao *et al.*, 1995, 1998; Holyoake *et al.*, 2001). Less research has addressed the effects of natural variation in cytoplasmic/mitochondrial genes on sperm traits that are assumed to influence sperm quality, and no studies to date have investigated possible interactions between cytoplasmic and nuclear genes on these traits. We note that there are reasons to believe such intergenomic interactions may affect sperm functioning (see Moore & Reijo-Pera, 2000). Moreover, there has been little empirical treatment investigating the potential contribution of cytoplasmic genes to the outcomes of sperm competition (with the exception of some research on the effects of the cytoplasmic bacteria, *Wolbachia*, on male fertility; Wade & Chang, 1995; Champion de Crespigny & Wedell, 2006). Sperm competition theory predicts that males with the most competitive ejaculates will have greatest fertilization success and that females may indirectly benefit from this competition between males because their sons will inherit paternal genes coding for highly competitive ejaculates or increased viability, and thus have higher fitness (Sivinski, 1984; Harvey & May, 1989; Keller & Reeve, 1995). However, if sperm traits that affect sperm competitive ability are encoded by mitochondrial genes (Birkhead *et al.*, 1999; Arnqvist & Rowe, 2005; García-González & Simmons, 2005; St John *et al.*, 2005), then this will limit the potential for females, whose ova are fertilized by males with the most competitive sperm, to receive indirect benefits. Adaptive evolution of male success in sperm competitiveness may also be significantly constrained because there can effectively be no selection on mitochondrial genes in males.

In this study, we examine effects of cytoplasmic and nuclear genes, and their interactions, on two sperm traits (viability and length) in the polygamous seed beetle, *Callosobruchus maculatus* (Coleoptera: Bruchidae). This was achieved by using repeated, introgressive backcrossing to create lines of beetles carrying orthogonal combinations of distinct cytoplasmic and nuclear lineages. We then conducted two separate assays. First, we utilized a fluorometric measure of sperm viability to examine differences in this trait between males of each line. This fluorometric measure of sperm viability is frequently utilized in contemporary sperm competition research (e.g. Hunter & Birkhead, 2002; Moore *et al.*, 2004; Snook & Hosken, 2004; García-González & Simmons, 2005). Second, we measured sperm length in all lines to assess whether there were any cytoplasmic effects on this trait.

Methods

Study species and stock populations

Callosobruchus maculatus is a pest of stored legumes. In the absence of food and water, and at 30 °C, 50% RH, mated females oviposit on the surface of seeds over a period of about 6 days (D. K. Dowling, personal observation; see Credland & Wright, 1989 for similar data at 27 °C and 70% RH). Larvae hatch from these eggs and burrow directly into the seed. The entire larval development phase is completed within the seeds and the emerging adults are immediately ready to reproduce.

Outbred stocks of five distinct *C. maculatus* wild-type populations were used for this experiment. Each of these stocks was originally collected from a distinct geographical area and the stocks have been maintained at large population sizes (>100 individuals) under controlled, laboratory conditions for at least 60 generations. These stocks are Brazil (BR), California, USA (CA), Yemen (YE) and two Nigerian stocks: Lossa (LO) and Oyo (OY). BR was obtained from Robert Smith at the University of Leicester and all other stocks from Peter Credland at the University of London. Since acquiring these stocks in April 2002, each has been cultured in large (1 L) glass jars at 30 °C, 50% RH on a 12L : 12D h light cycle and a 26–28 day (egg-to-adult development typically ranges from 20 to 28 days) discrete generation cycle. Each generation was propagated by approximately 300 mated individuals on 120 g of black-eyed beans, *Vigna unguiculata*. Each stock was cultured for approximately 15 generations in these standardized conditions before this study commenced.

Construction of 'cytonuclear introgression' lines

In February 2004, a single virgin female from each of the five stocks was mated to a male from the same stock and

then placed with him on 100 g of beans in a glass jar. These five females were effectively mitochondrial 'Eves'. Twenty to 30 full-sib virgin daughters were subsequently collected from each of these matings and separated into five groups of four to six daughters. Each of these groups was then placed with six to 10 males from one of the five stock populations in each of the 25 possible orthogonal combinations. For each combination of matings, the resulting offspring had inherited 100% of their cytoplasmic genes from their mother, 50% of their nuclear genes from their mother and 50% from their father. These offspring were used to found 25 corresponding 'cytonuclear introgression' lines. In each subsequent generation, 10 virgin daughters, from each of the 25 lines, were collected and backcrossed to six to 10 outbred males from the same stock population as their fathers (in jars with 120 g beans). In this way, 15 successive generations of backcrossing were used to disassociate each of the sampled cytoplasmic genomes (each genome derived from one of the five stocks) from the nuclear genome with which it was originally associated, replacing it with a new complement of nuclear genes (derived from one of the five stocks) (Table 1). In theory, after 15 generations of such backcrossing, >99.9% of the original nuclear genome of each 'cytonuclear introgression' line had been replaced, resulting in each of the cytoplasmic genomes expressed in five distinct and controlled nuclear backgrounds.

Many insects are infected by a range of maternally inherited, cytoplasmic bacteria such as *Wolbachia*. The presence of such bacteria can potentially confound the results of experiments designed to explore mitochondrial effects on fitness-related traits. Despite efforts to identify *Wolbachia* spp. in *C. maculatus* (Tuda *et al.*, 2006), there are currently no records of *Wolbachia* in this species. Infections have, however, been recorded in some congeners, such as *Callosobruchus chinensis* (Kondo *et al.*, 1999), where it is established that treatment of the ovipositioning substrate with the antibiotic, tetracycline hydrochloride, is an effective method to eliminate *Wolbachia* (Kondo *et al.*, 2002) and assumingly other such bacteria. We employed a similar protocol here. In the ninth generation, all beans used in the backcrossing process were thoroughly soaked in a 3% aqueous solution of tetracycline and then air-dried prior to their use as oviposition substrates. All beetles laid their eggs on these tetracycline-treated beans in this generation and the subsequent larvae developed inside and fed on these beans.

In the 15th generation, the number of individuals used in the backcrosses was increased (20 virgin females and 30 males on 120 g of beans) to ensure that sampling error would not create differences in nuclear DNA among the 'cytonuclear introgression' lines. From generation 15, the lines were kept as separate populations. From this time, the sperm viability assay described below was conducted in two blocks that were separated in time (using the offspring of parents collected within the first 3 days of hatching in both generation 15 and 16). To maintain similar densities of eggs per bean in both blocks, in generation 16, 35–40 nonvirgin females were placed with 40 males from each line. This increase in sampled females was necessary because the females here were nonvirgin and had thus already oviposited a substantial proportion of their eggs before being transferred to fresh jars to propagate the next generation.

In generation 17, further introgressive backcrossing was conducted by sampling 20 females per line and backcrossing these to 30 males from the outbred stock populations. All lines were then propagated by 300 individuals on 120 g beans from generation 18 onwards. The sperm length assay was conducted in generations 21–23 and included a maternal effects treatment (Young vs. Old mothers). In generation 21 (block 1), 35 pairs were collected on day 24 of the life cycle (Young mothers) and placed on 120 g beans. On day 30 of the life cycle (Old mothers), 30 pairs were collected and placed on 100 g beans. Individuals that were collected on day 30 had either a substantially longer egg-to-adult development, spent a longer time as adults or hatched from eggs that were oviposited from older mothers than individuals collected on day 24. On average, these individuals were older than those collected on day 24. Older mothers lay fewer eggs than younger mothers (Ouedraogo & Huignard, 1981; Dick & Credland, 1984) and therefore the number of individuals collected and the amount of ovipositioning substrate used was adjusted such that the larval density per bean was approximately the same in both Treatments and across Blocks. In generation 22 (Block 2), 29 pairs were placed on 100 g

Table 1 'Cytonuclear introgression' lines.

	c BR	c CA	c LO	c OY	c YE
n BR	n BR × c BR	n BR × c CA	n BR × c LO	n BR × c OY	n BR × c YE
n CA	n CA × c BR	n CA × c CA	n CA × c LO	n CA × c OY	n CA × c YE
n LO	n LO × c BR	n LO × c CA	n LO × c LO	n LO × c OY	n LO × c YE
n OY	n OY × c BR	n OY × c CA	n OY × c LO	n OY × c OY	n OY × c YE
n YE	n YE × c BR	n YE × c CA	n YE × c LO	n YE × c OY	n YE × c YE

Five distinct cytoplasmic genomes (c) were introgressed into five distinct nuclear (n) backgrounds. Cytoplasmic genes are inherited maternally and were introgressed into nuclear DNA from males of each stock population.

beans to propagate the Young treatment on day 25 of the life-cycle and 15 pairs were placed on 40 g beans in the Old treatment on day 32. In generation 23 (Block 3), 14 pairs were placed on 50 g beans in the Young treatment at day 23 and 14 pairs on 40 g beans on day 30 in the Old treatment.

Sperm viability assay

Collection of virgin males

In each block, we isolated 50 beans (infested with larvae) per cytonuclear introgression line in 'virgin chambers' at day 17 of the lifecycle. These chambers separate individual beans, which can then be checked regularly for emerging, virgin adults. Adults began emerging on day 20 and assays were subsequently conducted over a 7-day period per block (days 20–26). On each day, newly hatched virgin males were collected for that day's assays. All chambers were cleared of beetles each evening (at 19:30 hours) to ensure that the virgins collected the following day (at 07:00 hours) for the assays were less than 12 h old. Although this protocol controlled for adult age (all males were newly hatched virgins), individual males may have differed in juvenile development time. We thus included the day at which a given male hatched, counting from the day individuals commenced hatching from a specific line, in our analyses ('Hatching date'). This measure, then, will in part reflect juvenile development time and in part reflect the day at which the egg was oviposited from which a given male subsequently hatched.

Assay

In each sample, the seminal vesicles were dissected from two to five males per line in Hepes-buffered saline (HBS, pH 7.4 containing 10 mM Hepes, 154 mM NaCl, 7.2 mM KCl and 1.8 mM $CaCl_2$), placed in a 60 μL drop of HBS on a microscopic slide and the contents gently released and dispersed using dissecting scissors. A 15 μL drop of a master mix (containing 200 μL HBS, 12.5 μL propidium iodide and 25 μL of a 1 : 12 dilution of SYBR-14 : DMSO; Sperm Viability Kit, Molecular Probes, Eugene, OR, USA) was added to the slide and the solutions mixed with a dissecting needle. SYBR-14 and propidium iodide are dyes that differentially stain live (green fluorescence) and dead (red fluorescence) spermatozoa respectively. SYBR-14 is a membrane permeable, nucleic acid stain while propidium iodide stains damaged, permeable membranes. Two minutes later a cover slip was added and the slide examined at 200 × magnification with a Leica DMRX/E microscope (Leica Microsystems, Wetzlar, Germany) to locate sperm. At this point, the sample was visualized using a standard fluorescein excitation optical filter. Photographs were taken of 10 random fields using OPENLAB 3.0.9 (Improvision, Coventry, UK). On average, the total time taken from initiating dissections to completing the assay was 21.7 min (95% CI = 1.78, range 15–30 min) per sample.

Photographs were scored manually, summing the total number of green (live), red (dead) and doubly stained sperm. Doubly stained sperm comprised 21.6% of sampled sperm. Following convention (e.g. Collins & Donoghue, 1999; Bernasconi *et al.*, 2002), these sperm were considered inviable as they had lost their ability to resist propidium iodide uptake. Sperm viability was measured as the proportion of green (live) sperm per sample. Two different types of sperm were photographed within most samples: spermatozoa connected by their tails in bundles (mean number per bundle = 80.8, 95% CI = 75.0–86.6) and single, detached spermatozoa. Each photograph taken was classified into one of two categories: an image of detached sperm or an image of a sperm bundle. There were 757 photographs taken of sperm in bundles, capturing 61 365 sperm of 638 males in 179 samples (mean 3.58 males per sample) and comprising 31–39 samples per cytoplasmic lineage, 34–38 samples per nuclear lineage and 6–8 samples per cytonuclear line. In comparison, 1277 photographs were taken of detached sperm, capturing 17 840 sperm from 691 males in 194 samples (3.58 males per sample) and comprising 38–40 samples per cytoplasmic lineage, 37–40 samples per nuclear lineage and 6–9 samples per cytonuclear line.

Sperm length assay

Beetles were sampled over a 5 day period per Treatment per Block (days 20–24 of life-cycle). We isolated 50 beans (infested with larvae) per cytonuclear introgression line and collected virgins each morning using the same protocol as described for the previous assay. We collected both male and female virgins from each line, storing each sex separately on a temperature-controlled (30 °C) bench. We employed the following protocol to sample singleton sperm. A male was first mated to a female of the same line. The female's spermatheca was then dissected 90 min later, in diphosphate-buffered saline (DPBS; pH 7.4) containing 52.02 mM NaCl, 39.70 mM KCl, 0.54 mM $CaCl_2$, 1.18 mM $MgCl_2 \times 6H_2O$, 1.22 mM $MgSO_4 \times 7H_2O$, 1 mM glucose and 58.43 mM sucrose in 0.05 M PB. The contents of the spermatheca were squeezed onto a microscope slide in a 5 μL drop of DPBS. A cover slip was then placed on top of this and gentle pressure applied, so that all sperm, across all samples, were subsequently measured on the same plane. Sperm were then located under a Leica DMRX/E light microscope and photographs taken of eight separate and randomly selected sperm (with the sole criterion that they were undamaged) per sample at 400 × magnification.

Photographs of sperm were then measured using IMAGEJ V. 1.34 image analysis software (National Institutes of Health, Bethesda, MD, USA). The scale was calibrated in micrometres against a ruled grid at 400 ×

magnification. Then entire sperm length (from head to tail) was measured along the central longitudinal axis. To assess repeatability of the sperm length measure, approximately 5% of the sperm was measured twice, with the second measurement taking place at least 2 days after the first measurement, blind to the length of the first measure. Ten males were thus sampled per line and these were evenly distributed over both Treatments. In total, the lengths of over 2000 sperm were measured (eight sperm × 10 males × 25 lines).

Statistical analysis

Sperm viability

A greater proportion of the sperm in bundles was viable than detached sperm ($t_{2034} = 2.68$, $P < 0.01$, mean viability bundles = 0.73, 95% CI = 0.71–0.76, mean viability detached = 0.69, 95% CI = 0.68–0.71). Thus, there are many more sperm connected in bundles in seminal vesicles than detached sperm (61 365 in bundles, 17 840 detached) and sperm in bundles have higher viability than detached sperm. There are two possible mechanisms that could result in the incidence of two sperm types in our samples. First, these detached sperm may be at a different stage in the maturation/activation process. This is, however, unlikely because sperm mostly exist in bundles both within the male seminal vesicles and within the transferred spermatophore inside the female. They only appear in detached, singleton form inside the spermatheca of females (D.K. Dowling, A. Larkeson Nowostawski, personal observations; P. Eady, personal communications). Based on the above results and observations, it is much more likely that detached sperm are those that have accidentally become detached from bundles during the dissection/sampling process. If so, they will provide less relevant information for the subsequent analyses. This conclusion is further supported by there being no nuclear or cytoplasmic effects on these detached sperm (nuclear lineage: $F_{4,160} = 0.21$, $P = 0.932$; cytoplasmic lineage: $F_{4,160} = 0.23$, $P = 0.920$). For these reasons, we present only the analysis of sperm in bundles in this manuscript.

For each sperm type, cytoplasmic and nuclear effects on viability, together with hatching date and interactions were analysed in: (i) generalized linear models with binomial errors and logit links; and (ii) general linear models with arcsine-transformed proportions of viability. The unit of replication was the sample (i.e. mean sperm viability was calculated per sample). We only investigated the effect of second-order interactions because sample sizes per group for higher-order interactions were considered too small for the estimation of reliable parameter estimates. In generalized linear models, the number of live sperm per sample was the response variable and the total number of sperm the binomial denominator. Overdispersion was adjusted using Williams' correction within the 'Extrabinomial' procedure in GENSTAT V.7.0 (VSN International, Hemel Hempstead, UK). In all models presented, potential confounding parameters, such as 'block' (d.f. = 1, deviance ratio = 0.24, $P = 0.622$), 'time taken to analyse sample' (d.f. = 1, deviance ratio = 0.61, $P = 0.436$) and 'number of males per sample' (d.f. = 3, deviance ratio = 0.74, $P = 0.527$) had no effects on viability, and were therefore not included in the final analyses. A bootstrapping resampling procedure, using the residuals of the original model (1000 replicates), was employed to assess general linear models (ter Braak, 1987; Manly, 1997).

Sperm length

We calculated the mean sperm length per male sampled (mean of eight sperm). Effects of cytoplasmic and nuclear genes, together with maternal age, hatching date and block were examined on mean sperm lengths per male using a general linear model. As there were more factors in this analysis than in the sperm viability analysis, we reduced the full model by omitting nonsignificant second-order interactions.

Results

Sperm viability

Cytoplasmic genes influenced the viability of sperm in bundles. Indeed, the relative importance of cytoplasmic genes in determining variation in sperm viability was apparently greater than that of nuclear genes. First, a significant, or marginally nonsignificant, interaction between cytoplasmic and nuclear genes on viability can be inferred from both generalized and general linear models (Table 2). Some cytoplasmic lineages had higher sperm viability when expressed in some nuclear lineages, and lower viability when expressed in others. The matching of coevolved cytoplasmic and nuclear lineages (e.g. BR × BR) did, however, not appear to result in the highest sperm viability (Fig. 1). Moreover, a significant interaction between cytoplasmic lineage and hatching date on sperm viability was evident in both models (Table 2). Although there was no clear relationship between sperm viability and hatching date for most of the cytoplasmic lines, the BR lineage clearly decreased in viability with increasing age (Fig. 2). Retrospective linear regressions revealed that BR was the only cytoplasmic lineage in which there was a significant relationship between viability and hatching date.

There was an apparent discordance between the main effects of cytoplasmic lineage on viability of sperm in bundles, according to the model used (Table 2). We note, however, that the generalized linear model assesses significance by quantifying the change in deviance as terms are removed from a full model. Although such an analysis is effective in establishing the significance of interactions between factors, when such interactions

Table 2 The effects of nuclear genes, cytoplasmic genes, hatching date and their interactions on sperm viability for sperm sampled in bundles.

Source	d.f.	Deviance	Deviance ratio	*P* (model 1)	*P* (model 2)*	*P* (bootstrapped model 2)
Nuclear lineage	4	3.801	0.95	0.434	0.392	0.367
Cytoplasmic lineage	4	0.764	0.19	0.943	0.015	0.02
Hatching date	1	0.342	0.34	0.559	0.760	0.77
Cytoplasmic × nuclear	16	24.687	1.54	0.076	0.041	0.064
Nuclear × hatching date	4	1.444	0.36	0.837	0.662	0.678
Cytoplasmic × hatching date	4	11.311	2.83	0.023	0.018	0.024
Total	178	195.908				

The results were analysed with two models. Model 1 is a generalized linear model with binomial error and logit link, using sequential deletion for statistical inference. Model 2 presents probability values from a general linear model. Model 2 was also tested with a bootstrapping analysis (1000 replicates) based on the residuals.
*Shapiro–Wilks tests revealed that the residuals of this model did not differ significantly from normality. Bartlett's tests revealed no evidence for heterogeneity of variances among groups.

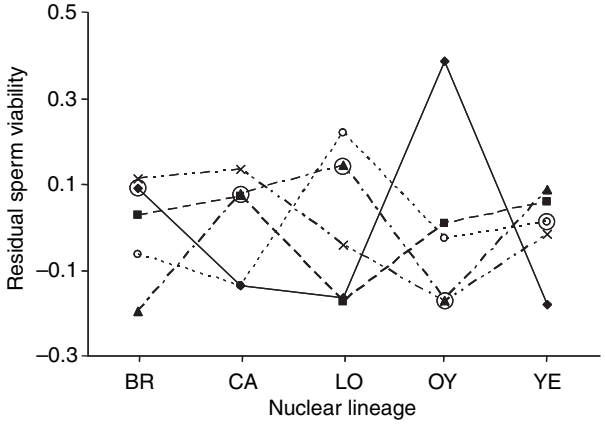

Fig. 1 Interaction plot of cytoplasmic × nuclear effect on viability for sperm in bundles (residuals of the general linear model in Table 2 excluding the cytoplasmic × nuclear lineage interaction). The five nuclear lineages are denoted along the horizontal axis. Each line represents one of the five cytoplasmic lineages: Brazil (BR), –◆–; California (CA), –■–; Lossa (LO), –▲–; Oyo (OY), –×–; Yemen (YE), –○–. Circles are drawn around matching cytoplasmic-nuclear combinations (e.g. BR × BR, etc).

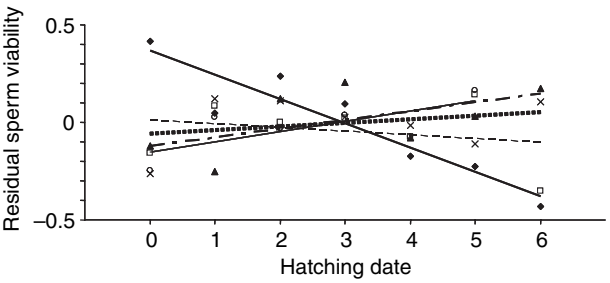

Fig. 2 Interaction plot of cytoplasmic lineage × hatching date on viability of sperm in bundles. Hatching date denoted on the horizontal axis. Each line illustrates the change in sperm viability (residuals of general linear model in Table 2 excluding the cytoplasmic lineage × hatching date interaction) of a specific cytoplasmic lineage according to the date at which males hatched. Brazil (BR), ◆ with trendline ——; California (CA), □ with line ----; Lossa (LO), ▲ with line ·····; Oyo (OY) × with line ·-·-·; and Yemen (YE), ○ with line ——.

occur it is not possible to appropriately assess the impact of main effects. This is because the relevant interactions must be first dropped from the full model prior to assessing the impact of main effects. In contrast, the general linear model is a type III model that assesses significance of each term while partitioning out variance due to all other terms in the model. Such a model can thus assess the significance of main effects given that interactions are accounted for. These are thus more appropriate for assessing main effects. The type III model revealed a significant effect of cytoplasmic lineage on sperm viability (Table 2). A bootstrapping test, based on the residuals of this model (ter Braak, 1987; Manly, 1997), confirmed that this model and associated parameter estimates were robust.

Sperm length

Mean sperm length across all sperm was 170.34 μm (n = 2008, SE = 0.17). The sperm length measurement was highly repeatable ($R = 0.99$, $n = 105$, $P < 0.001$). The repeatability in the lengths of the different sperm measured within each sampled male was also high ($R = 0.71$, $n = 251$, $P < 0.001$). There was a strong effect of nuclear lineage and a smaller effect of cytoplasmic lineage on sperm length (Table 3, Fig. 3). Moreover, there was a near-significant cytonuclear interaction on sperm length. Finally, there was a strong phenotypic maternal effect, with older mothers seemingly producing sons with longer sperm than younger mothers (Table 3, Fig. 4 – but see *Discussion* for interpretation). Individual weight (micrograms) was recorded for a sample of the focal males prior to the sperm length assay. There was no relationship between male weight and sperm length among these focal males ($r^2 = 0.01$, regression coefficient = 805.3, SE = 1840.4, $t_{29} = 0.44$, $P = 0.67$).

Table 3 Effects of nuclear genes, cytoplasmic genes, hatching date, maternal age, block and their interactions on sperm length.

Source	d.f.	Sum-of -squares	*F*-ratio	*P*
Nuclear lineage	4	1771.44	18.31	<0.001
Cytoplasmic lineage	4	330.46	3.42	0.01
Hatching date	1	76.74	3.17	0.076
Maternal age	1	402.39	16.64	<0.001
Block	2	2108.12	43.59	<0.001
Cytoplasmic × nuclear	16	641.97	1.66	0.056
Total	250			

Shapiro–Wilks tests revealed that the residuals of this model did not differ significantly from normality. No evidence was found to suggest heterogeneity of variances among groups.

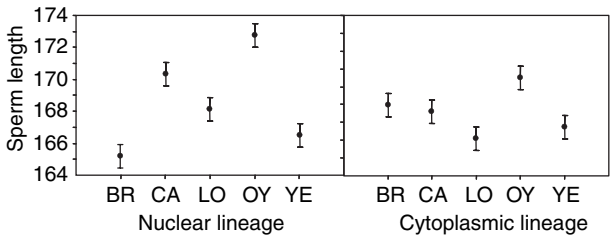

Fig. 3 Mean sperm length (adjusted least squares means of general linear model in μm ± 1 standard error) for each nuclear and cytoplasmic lineage. Means are based on 50 males per lineage, with the exception of the Brazil (BR) nuclear lineage ($n = 51$) and Yemen (YE) cytoplasmic lineage ($n = 51$).

Fig. 4 Maternal age effect on sperm length (adjusted least squares means of general linear model, in μm ± 1 standard error) of focal males. Sample sizes are $n = 125$ for Young mothers and $n = 126$ for Old mothers.

Correlations between sperm viability and length

Mean sperm viability was positively related to mean sperm length across the cytoplasmic lineages ($r^2 = 0.76$, regression coefficient = 0.011, SE = 0.004, $t_5 = 3.04$, $P = 0.056$, Fig. 5a) and negatively related to sperm length across the nuclear lineages ($r^2 = 0.73$, regression coefficient = −0.011, SE = 0.004, $t_5 = -2.87$, $P = 0.064$, Fig. 5b). Both of these relationships were near significant.

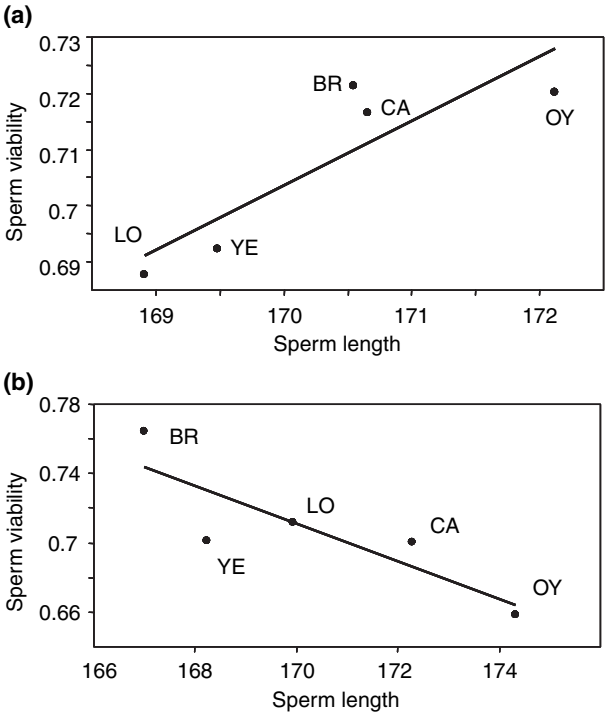

Fig. 5 (a) Mean sperm length vs. mean sperm viability across the five cytoplasmic lineages. (b) Mean sperm length vs. mean sperm viability across the five nuclear lineages.

Discussion

We assessed whether natural variation in cytoplasmic genes influenced two sperm traits. We present several lines of evidence that cytoplasmic genes indeed affect variation in both sperm viability and length. First, sperm viability varied across five distinct cytoplasmic lineages. Furthermore, an interaction between cytoplasmic lineage and hatching date had significant effects on sperm viability, and an interaction between cytoplasmic and nuclear genes also appeared important. In contrast, we found no significant main effect of nuclear lineage on viability, and no interaction between nuclear lineage and hatching date. This suggests a more important contribution of cytoplasmic genes than nuclear genes to variation in sperm viability in *C. maculatus*. Sperm length varied across both cytoplasmic and nuclear lineages and there was also a tendency of a cytonuclear interaction on sperm length. Based on a comparison of *F*-values, however, nuclear lineage had a much stronger effect on sperm length than did cytoplasmic lineage. There are good reasons to assume that the cytoplasmic effects on

sperm traits revealed in our assays reflect underlying variation in mitochondrial genes. This is primarily because cytoplasmic bacteria, such as *Wolbachia*, are unlikely to have contributed to the observed patterns (see *Methods*).

These results have potential implications for sperm competition theory, in particular, the sexually selected sperm hypothesis that predicts that sperm traits will evolve adaptively (Sivinski, 1984; Harvey & May, 1989; Keller & Reeve, 1995). The quality of a sperm is likely to be influenced by several, potentially inter-related, traits such as length, viability, motility and longevity. If multiple sperm traits are encoded by cytoplasmic genes, then this may result in constraints on the scope for adaptive evolution of sperm quality and for post-mating sexual selection by adaptive 'sperm choice' by females (Keller & Reeve, 1995). Here, we found cytoplasmic effects on two sperm traits. In particular, the effect of cytoplasmic lineage on sperm viability appeared to be greater than nuclear lineage. Recently, there is accumulating evidence, within- and across-species, that sperm viability may be important in determining the outcome of sperm competition (e.g. see Hunter & Birkhead, 2002; García-González & Simmons, 2005). The effect of sperm length on male fertilizing ability is more equivocal; there are reports of positive associations between sperm length and indices of sperm competition (Gage, 1994; Byrne *et al.*, 2003), negative associations (Gage & Morrow, 2003) and no associations (Simmons *et al.*, 2003; Gage *et al.*, 2004; see reviews by Hosken, 2003; Snook, 2005). Furthermore, mutations in mtDNA have been implicated in reductions in sperm motility in humans (e.g. Ruiz-Pesini *et al.*, 2000) and sperm motility may also be an important component of sperm competitive ability (see Birkhead *et al.*, 1999). Given our results, there may also conceivably be cytoplasmic effects on sperm motility in *C. maculatus*. We were, however, unable to measure motility because the sperm of *C. maculatus* were essentially immotile outside of the female reproductive tract (i.e. in saline buffers).

The potential for male sperm traits to evolve depends, in part, on underlying additive genetic variation. We found a large main effect of nuclear lineage on sperm length, but no detectable main effect on sperm viability. Several previous studies have investigated the quantitative genetics of sperm length. Ward (2000) and Morrow & Gage (2001) determined that sperm length in the yellow dung fly (*Scathophaga stercoraria*) and a cricket (*Gryllus bimaculatus*), respectively, was heritable and probably X chromosome linked. Simmons & Kotiaho (2002) found that sperm length was heritable in the dung beetle (*Onthophagus taurus*), and almost all the genetic variation was additive. Birkhead *et al.* (2005) found that sperm head, midpiece and flagellum length were heritable in the zebra finch (*Taeniopygia guttata*), but that maternal genetic effects (presumably mitochondrial effects) also existed. The above findings are largely consistent with our finding of a strong nuclear effect and a weaker, but significant cytoplasmic effect on sperm length in *C. maculatus*. In contrast, the genetic architecture underlying the sperm viability phenotype in *C. maculatus* is seemingly less consistent with other taxa. For instance, it was recently demonstrated that there is significant additive genetic variation for sperm viability in field crickets (Simmons & Roberts, 2005). However, Moore *et al.* (2004) examined the quantitative genetics of ejaculate characteristics in the cockroach (*Nauphoeta cinerea*) and found that narrow-sense heritabilities were high for all measured components of ejaculate quality (including sperm numbers, spermatophore mass, sperm ampulla mass and testes mass) except sperm viability. The broad-sense heritability of sperm viability was about double that of the narrow-sense heritability, indicating that nonadditive effects, such as environmental or maternally inherited effects, might be important for this trait. This is consistent with our finding of no significant main effect of nuclear lineage, but a significant cytoplasmic effect on sperm viability in *C. maculatus*. Thus, at least in seed beetles and cockroaches, sperm viability may be a component of the ejaculate that is evolutionarily constrained by its genetic architecture.

The results of this study suggest that interactions between cytoplasmic and nuclear genes influence sperm viability and potentially sperm length. In other words, the sperm viability and length of a particular cytoplasmic lineage depended, in part, on the nuclear background in which it was expressed. The detection of such an interaction on sperm quality is not surprising given that previous research has identified contributions of both genomes to sperm and energy production (see Moore & Reijo-Pera, 2000). However, we note that such epistatic interactions may further reduce the potential for indirect benefits to females who mate with males with competitive ejaculates. We also found a significant interaction between cytoplasmic lineage and hatching date on sperm viability. In particular, one of the cytoplasmic lineages (BR) steadily decreased in viability with hatching date whereas the other lineages did not. As mentioned earlier, the term hatching date in this study potentially encompasses two age-related factors. On average, offspring that hatch later have hatched from eggs oviposited by older mothers and/or have taken longer to develop from egg-to-adult. There is an accumulating body of evidence demonstrating that mutations in mtDNA, and indeed natural variation in cytoplasmic genes, may affect patterns of ageing in different organisms (James & Ballard, 2003; Samuels, 2004; Kujoth *et al.*, 2005; Trifunovic *et al.*, 2005). It is thus possible that some mtDNA haplotypes essentially age faster than others, with detrimental effects on sperm viability. Our results are, at least, consistent with this interesting possibility.

Our findings of cytoplasmic effects on sperm traits are congruent with some other recent findings. For instance, known mtDNA mutations are associated with reductions

in sperm motility in humans (Kao *et al*., 1995; Ruiz-Pesini *et al*., 2000; Holyoake *et al*., 2001). Additionally, sperm competitive ability in domestic fowl (*Gallus gallus domesticus*) appears to be largely determined by a maternally inherited element, potentially cytoplasmic genes (Froman *et al*., 2002). Here, we find effects of cytoplasmic genes on sperm viability and length in an insect. Unlike previous studies examining human sperm quality, the effects we find are unlikely to be caused by deleterious mtDNA mutations that drastically reduce male fertility because all the distinct cytoplasmic types used in this study are easily cultured with correspondingly high fitness. Rather, we suggest that our results reflect more subtle differences in sperm quality due to natural variation in cytoplasmic genes. Nonetheless, these differences may translate into consistent differences in sperm competitive ability and hence male fertilization success under sperm competition.

Mean sperm viability was positively associated with mean sperm length across cytoplasmic lineages. This suggests that the same cytoplasmic genes may encode both sperm traits. A mechanism via which this could occur is through differential metabolic outputs across the different lineages. If the production of large numbers of high-quality sperm is energetically costly (see Dewsbury, 1982; Van Voorhies, 1992; Pitnick & Markow, 1994; Pitnick, 1996; Olsson *et al*., 1997), then metabolic output across the cytoplasmic lineages may covary with sperm quality. We know of no tests of this possibility, although male *C. maculatus* produce ejaculates comprising about 7% of their body weight (Savalli & Fox, 1998) and 46 000 sperm (Eady, 1994) and thus it is likely that the energetic investment associated with this production is substantial. The reverse pattern was observed across nuclear lineages, with the tendency of a negative association between the two sperm traits. Such negative correlations between sperm traits have been observed previously in the zebra finch (Birkhead *et al*., 2005). A negative association between sperm traits may arise if the alleles for these traits were antagonistic pleiotropic, in linkage disequilibrium, or if the adaptive potential of one of the sperm traits was constrained due to it being primarily encoded by cytoplasmic genes (Birkhead *et al*., 2005). If mutations in mtDNA accrued within the cytoplasmic lineage that resulted in decreased performance of this sperm trait, the nuclear genome may potentially compensate for this by adaptively increasing the performance of another sperm trait that is primarily determined by nuclear genes.

Finally, we found that mothers from the 'Old treatment' produced sons with longer sperm. It was surprising to find seemingly maternal phenotypic effects on sperm length, considering (i) that there was no detectable relationship between the mass of the focal males and sperm length in this study; and (ii) as far as we are aware, no other studies have found such an effect. Furthermore, assuming that longer sperm is more energetically costly to produce and competitively superior (an assumption that is not verified in this species), the direction of this pattern was even more surprising given that mothers from the 'Old treatment' are in poorer phenotypic condition than those from the 'Young treatment' (Fox & Dingle, 1994; Fox *et al*., 2003). The most plausible explanation for this observed result was that the larval density (eggs per bean) varied consistently between the maternal age treatments. Although we attempted to regulate larval density at three to four eggs per bean across both maternal age treatments (see *Methods*), we did not quantify this parameter and thus it is possible there were small but consistent differences in larval density between the treatments. Such a density-dependent effect on sperm morphology would, nonetheless, be novel if true and consistent with studies by Gage (1995), who found a similar density-dependent effect on numbers of sperm produced in the moth (*Plodia interpunctella*), and Green (2003), who found that sperm length increases with body mass among young male rove beetles (*Aleochara bilineata*). However, further empirical experimentation is required to establish the exact nature of these environmental effects on sperm length.

In conclusion, we found sizeable cytoplasmic effects on viability of sperm. We also found a significant cytoplasmic effect on sperm length, but the nuclear effect was a stronger determinant of this phenotypic trait. These findings suggest that the adaptive evolution of at least some sperm traits, such as viability, may be constrained by a genetic architecture that does not permit a genetic response to selection by sperm competition in males.

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