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A comparison of nuclear and cytoplasmic genetic effects on sperm competitiveness and female remating in a seed beetle

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Abstract

It is widely assumed that male sperm competitiveness evolves adaptively. However, recent studies have found a cytoplasmic genetic component to phenotypic variation in some sperm traits presumed important in sperm competition. As cytoplasmic genes are maternally transmitted, they cannot respond to selection on sperm and this constraint may affect the scope in which sperm competitiveness can evolve adaptively. We examined nuclear and cytoplasmic genetic contributions to sperm competitiveness, using populations of *Callosobruchus maculatus* carrying orthogonal combinations of nuclear and cytoplasmic lineages. Our design also enabled us to examine genetic contributions to female remating. We found that sperm competitiveness and remating are primarily encoded by nuclear genes. In particular, a male's sperm competitiveness phenotype was contingent on an interaction between the competing male genotypes. Furthermore, cytoplasmic effects were detected on remating but not sperm competitiveness, suggesting that cytoplasmic genes do not generally play a profound evolutionary role in sperm competition.

Introduction

Females of many species mate multiply, and the ensuing competition between males for fertilizations often continues after copulation, via sperm competition. This competition is intense and any phenotypic variation in sperm competitiveness among males will be subject to strong sexual selection. Thus, sperm competition is widely acknowledged to be a profound evolutionary force (see Parker, 1970; Birkhead & Møller, 1998; Simmons, 2001) and, indeed, much of sperm competition theory is grounded on the assumption that sperm competitiveness evolves adaptively (e.g. Sivinski, 1980, 1984; Harvey & May, 1989; Curtsinger, 1991; Keller & Reeve, 1995; Yasui, 1997). Accordingly, the results of certain studies in which the study populations were subjected to experimentally enforced evolution, under conditions of monogamy and polyandry, support the premise that sperm competitiveness evolves (Hosken *et al.*, 2001; Tilszer *et al.*, 2006).

However, various constraints exist that may reduce the ability of a given component of sperm, or sperm competitiveness as a whole, to respond effectively to the selection generated via sperm competition. The definitive constraint would be a lack of additive genetic variance underlying sperm competitiveness. To date, only a few studies have examined the underlying genetics of sperm competitive ability, in a heuristic sense (as determined by the competitive ability of the ejaculate as a whole), and these have generally found low levels of additive genetic variance for this trait (Hughes, 1997; Simmons, 2003; Friberg *et al.*, 2005; but see Radwan, 1998). On the other hand, studies that have focused on specific components of sperm associated with production (e.g. numbers), morphology (e.g. length) and performance (e.g. motility, viability) have typically found higher levels of additive genetic variation for these components (Ducrocq & Humblot, 1995; Rege *et al.*, 2000; Froman *et al.*, 2002; Simmons & Kotiaho, 2002;

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Moore *et al.*, 2004; Birkhead *et al.*, 2005; Simmons & Roberts, 2005). This indicates that there is indeed reasonable potential for these specific 'sperm traits' to evolve adaptively, but constraints may nonetheless exist on the overall evolvability of the 'sperm competitiveness' phenotype.

There are various factors that may contribute to lowering the amount of additive genetic variance underlying, or the heritability of, the sperm competitiveness phenotype and these factors may thus constrain the potential for this trait to respond adaptively to selection. For instance, negative genetic correlations are likely to exist between some of the traits that contribute to overall sperm competitiveness. Such correlations, which result either from pleiotropy or linkage disequilibrium, are seemingly common among sperm traits (Moore *et al.*, 2004; Birkhead *et al.*, 2005) and are likely to impede an optimal response to selection by the particular traits involved. Additionally, sperm competitiveness is an unusual trait given that the phenotype, at least in *Drosophila melanogaster* and *Callosobruchus maculatus*, resulting from a particular male genotype is contingent on the genotypes of both the males with which he directly competes (Clark *et al.*, 2000) and/or the female with which he mates (Wilson *et al.*, 1997; Clark & Begun, 1998; Clark *et al.*, 1999). Thus, sperm competitiveness is effectively an interacting phenotype (Moore *et al.*, 1997; Moore & Pizzari, 2005). As a result, the realized units of selection on this trait essentially become pairs of interacting individuals or even male–male–female triplets (Clark *et al.*, 1999, 2000), rather than single individuals, which may in turn constrain an adaptive response to selection. Further constraints will arise if the sperm competitiveness phenotype is highly sensitive to environmental heterogeneity because this will increase the amount of residual phenotypic variance relative to additive genetic variance, thus reducing the heritability and a short-term response to selection (Houle, 1992). Inevitably, genotype-by-environment interactions underlying the sperm competitiveness phenotype will also complicate a response to selection (Hunt *et al.*, 2004). Finally, genetic constraints on the response to selection may exist if a substantial part of the sperm competitiveness phenotype is encoded by genes that are exclusively maternally transmitted, such as mitochondrial/cytoplasmic genes (Frank & Hurst, 1996; Pizzari & Birkhead, 2002; Gemmell *et al.*, 2004).

Indeed, several authors have reasoned that some of the phenotypic variation observed in a number of sperm traits should be explained by polymorphism in mitochondrial genes (Frank & Hurst, 1996; Moore & Reijo-Pera, 2000; Gemmell & Allendorf, 2001; Pizzari & Birkhead, 2002; Gemmell *et al.*, 2004; Zeh, 2004; Dowling *et al.*, 2007a). The logic here is that sperm motility, at least in some species (Mitchell *et al.*, 1976; Bigliardi *et al.*, 1970; Báo *et al.*, 1992; but see Baccetti *et al.*, 1973; Perotti, 1973; Werner *et al.*, 1999), is achieved using energy generated by the mitochondria located within the sperm flagellum. Moreover, given the high energetic demand required to produce large numbers of viable sperm (Dewsbury, 1982; Kenagy & Trombulak, 1986; Gage, 1991; Van Voorhies, 1992; Pitnick & Markow, 1994; Pitnick, 1996; Olsson *et al.*, 1997), and the essential role of mitochondrial genes in energy production, polymorphism in mitochondrial genes may translate into the differential ability of mitochondrial haplotypes to produce large numbers of high-quality, viable sperm. Empirical studies have now implicated a likely cytoplasmic/mitochondrial genetic component to phenotypic variation in sperm motility (Ruiz-Pesini *et al.*, 2000; Froman *et al.*, 2002), sperm viability (Dowling *et al.*, 2007a) and sperm length (Birkhead *et al.*, 2005; Dowling *et al.*, 2007a) in some species.

The consequences of this cytoplasmic genetic component to sperm morphology and performance may be important given that cytoplasmic (including mitochondrial) genes are maternally inherited in most anisogamous species and there is, therefore, no selection on these genes in males (Pizzari & Birkhead, 2002; Zeh, 2004). Thus, mutations accruing in the mitochondrial genome that have male-specific deleterious effects on fitness (such as on sperm competitiveness) can theoretically be maintained in a population if these mutations are neutral when expressed in females (Frank & Hurst, 1996). This sex-specific selective sieve is predicted to result in the accumulation of a greater amount of cytoplasmic genetic variation for male, relative to the female, homologues of sexually dimorphic traits (Frank & Hurst, 1996). Furthermore, if mtDNA mutations with adverse effects in males are beneficial when expressed in females, selection will act to fix these alleles despite their negative pleiotropic effects on male fitness (Chippindale *et al.*, 2001; Rand *et al.*, 2001; Arnqvist & Rowe, 2002, 2005). Ultimately, the sex-specific selection of mtDNA may result in constraints on the potential for male-limited traits, such as sperm performance, that have a cytoplasmic genetic component to respond efficiently to selection (Birkhead *et al.*, 1999; Arnqvist & Rowe, 2005; García-González & Simmons, 2005; Dowling *et al.*, 2007a).

Given that cytoplasmic genes are known to affect the expression of some sperm performance and sperm morphological traits (Ruiz-Pesini *et al.*, 2000; Dowling *et al.*, 2007a), it is plausible that they will have concomitant effects on the overall sperm competitiveness phenotype, thus placing further constraints on the scope for the adaptive evolution of this trait. At the very least, a cytoplasmic genetic component to sperm competitive ability will increase the residual variation relative to additive genetic variation, thus reducing heritability. However, in the most extreme scenario, cytoplasmic mutations or haplotypes resulting in low sperm competitiveness, and hence male fertility, may accumulate to high-enough frequencies within a population

(Ruiz-Pesini *et al.*, 2000) to potentially reduce population viability and ultimately drive that population to extinction (see Gemmell & Allendorf, 2001; Gemmell *et al.*, 2004). Needless to say, the potential for any such constraints to occur within a given species rests on the untested assumption that the cytoplasmically encoded phenotypic variation observed among sperm traits has an important overall contribution to the sperm competitiveness phenotype.

Here, we assess this assumption by comparing the relative role of cytoplasmic and nuclear genes on overall sperm competitiveness in the polygamous seed beetle, *C. maculatus* (Coleoptera: Bruchidae). To increase the likelihood of finding an effect, we used lines of beetles carrying orthogonal combinations of five distinct cytoplasmic and nuclear lineages that are derived from different populations. A previous study identified cytoplasmic genetic effects on both sperm viability and sperm length across these same lines (Dowling *et al.*, 2007a). Sperm competitiveness was assessed here using a sperm displacement assay, in which focal males of each line were given the opportunity to mate with once-mated females and, when successful in coercing females to remate, the resulting fertilization success of these males was scored. Furthermore, the fertilization success of the focal males of each line was tested in separate contexts. Focal males competed against males possessing either the same cyto-nuclear genotype as themselves or a novel genotype derived from a distinct population (Zaire), for fertilizations with females of either their own cyto-nuclear genotype or the novel genotype. The design of this experiment also enabled us to examine genetic effects on female remating rates, as a substantial proportion of females in the assay did not remate.

Methods

Construction of 'cytonuclear introgression' lines

Outbred stocks of five distinct *C. maculatus* populations were used to generate lines fixed for orthogonal combinations of distinct cytoplasmic and nuclear lineages. These stocks were: Brazil (BR), California (CA), Yemen (YE) and two Nigerian stocks: Lossa (LO) and Oyo (OY). Each of these stock populations was originally collected from a distinct geographical area and then maintained at large population sizes (>100 individuals) under controlled laboratory conditions for at least 60 generations. We acquired these laboratory stocks in April 2002 and have since cultured them at 30°C, 50% relative humidity (RH), without food or water, on a 12 : 12 h light : dark cycle and a 26- to 28-day discrete generation cycle. Each generation was propagated by approximately 300 mated individuals on 120 g of black-eyed beans, *Vigna unguiculata*, for approximately 15 generations in these standardized conditions before this study commenced. (See Dowling *et al.*, 2007a, for further details on these populations.)

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. 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 inherited 100% of their cytoplasmic genes from their mothers, 50% of their nuclear genes from their mothers and 50% from their fathers. These offspring were used to found 25 corresponding 'cytonuclear introgression' (CN) 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). In theory, after 15 generations of such backcrossing, >99.9% of the original nuclear genome of each CN line had been replaced, resulting in each of the cytoplasmic genomes being expressed in five distinct and controlled nuclear backgrounds. Although infections with the cytoplasmic-inherited bacteria *Wolbachia* have not been detected previously in *C. maculatus* (Tuda *et al.*, 2006), we treated all lines with tetracycline hydrochloride at generation 9 to eliminate any bacterial infections present (see Dowling *et al.*, 2007a).

Following generation 15, CN lines were maintained as separate populations, with further backcrossing conducted in generations 18 and 29. In generations 15, 18 and 29, 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 CN lines. Otherwise, population sizes were maintained at 35–40 pairs on 120 g beans per line in generation 16, approximately 100 pairs on 120 g beans in generations 17 and 19, and approximately 150 pairs on 120 g beans in generations 20–28. In generations 30 and 31, each line was propagated by 40 pairs on 120 g beans. (See Dowling *et al.* 2007a for further details of the introgression protocol.)

Culturing of Zaire stock population

The Zaire (ZA) stock population is derived from a distinct African geographic location and has been maintained

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within our laboratory since April 2002 under the standardized conditions described above. At generation 29 of the CN lines, we established a parallel and synchronous population of the ZA stock, propagated by approximately 150 pairs on 200 g beans, and subsequently propagated by 100 pairs on 300 g beans at generations 30 and 31. Thus, the ZA stock was cultured at the same larval density as the CN lines in generations 30 and 31.

Experimental design

Outline

The following assay was designed to test the fertilization success of males of each CN line when mating to once-mated females. Specifically, CN males from each line either competed against males of their own CN genotype or against males of a novel genotype (ZA nuclear-/cytotype) for fertilizations in females of either their own CN or the ZA genotype. Paternity was assessed using the 'sterile male' technique (see below), a reliable method of paternity assignment in *C. maculatus* (Eady, 1991).

Collection of virgin beetles

The experiment was conducted in two blocks, using offspring collected from generations 30 (block 1) and 31 (block 2). In each block, we isolated 75 beans per CN line and 400 beans from the ZA stock (all beans infested with larvae) in 'virgin chambers' at day 18 of the life cycle. These chambers isolate individual beans in cells, which are then checked regularly for emerging virgin adults. We used these chambers, stored at 30°C, to collect the beetles required for the experimental assay. Females, and males that were to mate with the females first (hereafter referred to as 'P1 males'), were collected from either the CN lines or the ZA stock. All focal males, that were to be given the opportunity to mate with the once-mated females (hereafter referred to as 'P2 males') were collected from the CN lines.

Adults began emerging from beans on day 20. On the evening of day 21 (and each subsequent evening) all chambers were examined and any eclosed adults discarded. This ensured that all beetles used in the assay were less than 24 h old at the time of collection. All beetles required for the assay were collected during the daytime between days 22 and 24. To ensure that all beetles were virgin, individuals were only collected when found either alone or with other beetles of the same sex in their associated cell. Twelve males were collected from each CN line on day 22 (CN P1 males). An additional 44 males were collected from each CN line (CN P2 males) over days 22–24. Two hundred and sixty five males were collected from the ZA population over days 22–23 (ZA P1 males) and 265 females (ZA females) over days 23–24, while 12 females were collected from each CN line (CN females) on day 23. All collected males were stored according to class [P1 (CN, ZA) and P2 (CN) males] and their day of collection at room temperature, with standardized densities per dish. All females were stored in individual Petri dishes at room temperature.

Matings

All beetles to be used in the matings were placed in a 30°C, 50% RH climate chamber at 07:00 hours on day 25. Between 08:30 and 10:00 hours, all P1 males were irradiated with a dose of 70 Gy using a caesium-137 source (Division of Biomedical Radiation Sciences, Uppsala University). This dosage effectively renders *C. maculatus* males 99.3% sterile for at least the first 8 h post-irradiation (see Fig. S1), without noticeably affecting behaviour or longevity. The sterilized males mate readily and their sperm fertilize the ova, but these ova do not hatch (Boorman & Parker, 1976; Eady, 1991).

Then, 10 virgin females per CN line (10×25 CN lines = 250 females) and 250 ZA virgin females were mated to these P1 males over the next 8 h at 30°C, according to the following schedule involving four combinations (mating classes) of female and P1 male genotypes (Table 1). All females were mated individually in separate dishes, with spot-checks each minute to ensure each mated only once. Mating is unambiguous in *C. maculatus* and males were discarded once a mating was completed. For each of the 25 CN lines, we mated 10 CN females and 10 corresponding ZA females. The 10 females from each CN line were divided into two groups. Half of these were mated to a P1 male from the same CN line, while the other half were mated to a P1 male from the ZA stock. In the same way, the 10 corresponding ZA females were divided into two groups, with half mated to a P1 male from the corresponding CN line and half mated to a P1 male from the ZA stock (Table 1). This mating schedule was fulfilled using a staggered approach, with the females of each CN line, and associated ZA females, mated individually over a 15- to 20-min period. This approach allowed us to control the time interval between mating and the subsequent exposure to P2 males for each female. All CN females were 3 days old when mated, ZA females either 2 or 3 days (on average, three-fifths per mating class per CN line were 3 days old), CN P1 males 4 days, and ZA P1 males 3 or 4 days old (on average, three-fifths per mating class per CN line were 3 days).

Table 1 Schedule for matings and rematings, showing the four different combinations (mating classes), and numbers, of genotypes assayed per CN line.

		Matings – day 25	Rematings – days 26 and 27
Mating class	♀ genotype	♂ (P1) genotype – sterile	♂ (P2) genotype – viable
1	5 CN females	5 CN males	10 CN males
2	5 ZA females	5 CN males	10 CN males
3	5 CN females	5 ZA males	10 CN males
4	5 ZA females	5 ZA males	10 CN males

Female and P1 male age at mating was further controlled in the subsequent statistical analyses.

Rematings

On day 26, each mated female was placed with two corresponding CN P2 males (see Table 1) in an individual dish, at 30°C, approximately 24 (±2) h after the initial P1 mating. At the same time, each female was provided with a single 'inducement' bean upon which to oviposit, as females that are provided with ovipositioning substrate are more receptive to remating (Eady *et al.*, 2004). All P2 males were between 3 and 5 days old and putatively viable (i.e. not irradiated). Two-fifths of the P2 males used per mating class per CN line were 3 days, two-fifths 4 days, and one-fifth 5 days old. Each female was monitored for 1 h with regular spot-checks (every 1–2 min). Once a female had remated, the inducement bean was placed in a labelled vial, the mated male was discarded and 14 new beans were added to the dish upon which the female could oviposit. Beetles that did not remate during this 1-h period were separated and the females retained in the dish containing the inducement bean. All P2 males that did not mate on day 26 were recycled for use on day 27.

On day 27, each female that had not already remated was provided with a second inducement bean and again exposed to two corresponding CN P2 males in a dish, approximately 48 (±2) h after the P1 mating. Again, each female was monitored carefully over a 1 h period. As above, remated females were each provided with 14 beans, the two inducement beans retained in a separate vial, and males discarded.

Females were discarded from dishes 5 days after remating and the 14 beans retained. Under the standardized conditions in which these beetles are maintained, we expect that most eggs were laid by day 5 of oviposition (D.K. Dowling, pers. obs.; see Credland & Wright, 1989, for similar data for beetles stored at 27°C). This is particularly likely given that females were already 3 or 4 days old when commencing egg-laying.

Data collection

The number and paternity of all eggs oviposited on the 14 beans per female were scored 12–18 days after the rematings. Assigning paternity is straightforward as eggs fertilized by sterile P1 males do not hatch and remain transparent whereas those fertilized by viable P2 males hatch and become white and opaque as the larvae burrow into the seed and leave seed shavings inside the egg. As irradiation is so effective at causing sterility (see Fig. S1; Eady, 1991), and as egg hatch rates are consistently high across *C. maculatus* strains cultured under these standardized conditions (Arnqvist *et al.*, 2004), the proportion of hatched eggs (white eggs/total eggs) provides a reliable estimate of the fertilization success of a P2 male here. The fecundity of doubly mated females was calculated as the total number of eggs oviposited on the 14 beans.

The number of eggs on the inducement beans was also scored for females that remated and used as a covariate in the statistical analysis of P2 male fertilization success. The colour of these eggs was also checked to verify that they had been sired by sterile P1 males.

Statistical analyses

We note that P1 male type and female type were both treated as binary factors in all the statistical analyses. Specifically, P2 males competed either against P1 males of their own CN genotype with which they had 'coevolved', or against 'novel' P1 males of the ZA stock (thus classified 'coevolved' or 'novel'), for rematings with CN females (coevolved) or ZA females (novel).

Remating

Cytoplasmic and nuclear effects on the ability of P2 males to induce remating were analysed using a generalized linear model with binomial error and logit link. The response variable was whether the female remated (0/1), with a binomial denominator of 1, and the unit of replication was the female. Females had the opportunity to remate after 24 h and those that did not were given a second opportunity after 48 h. We analysed remating propensity for the whole data set (i.e. at 48 h) by combining the remating data at the 24- and 48-h exposures. We also examined the remating propensity of females at the 24-h exposure in a separate analysis.

The cytoplasmic and nuclear lineage of the P2 male, P1 male type, female type, P1 male age, female age and block were entered as fixed factors. The main effects and interactions between fixed factors were examined, and statistical significance of each term assessed by examining the probability associated with the change in deviance in the model when that term was dropped from a full model (i.e. G). No third-order interactions were significant, nor interactions involving block, P1 male age or female age. Thus, to reduce the total number of terms in the 'working' full models, we excluded these interactions in the subsequent analysis. Thus, the working full model contained all possible main effects and interactions between nuclear and cytoplasmic lineages, and between the genetic lineages and P1 male and female types. The statistical significance of each term was then assessed, as above. We note that main effects cannot be dropped if involved in interactions in a generalized linear model and, thus, interactions involving a given main effect were dropped from the model before assessing the significance of that particular main effect. For simplicity of presentation, only the main effects and significant interactions are presented. These interactions are illustrated in the figures using mean values calculated from the raw data. However, we note that the predicted mean values, generated from the generalized linear model and then

back-transformed to the original scale, match these raw means closely.

The advantage of this experimental design is that it enables us to determine whether variation in cytoplasmic or nuclear genes among the five lineages affects female remating rates. Moreover, the design allows us to assess whether these patterns of variation differ according to whether P2 males are competing against/interacting with familiar P1 male/female genotypes that they have coevolved with or novel (ZA) genotypes. However, the drawback of the design is that it is difficult to disentangle whether the genes causing the variation among lineages are expressed by the P2 male or the interacting P1 males/females as the cyto-nuclear genetic combinations are partly confounded (i.e. P2 male cyto-nuclear genotypes are tested against identical P1 or female genotypes in 50% of cases). We expect that P2 males, P1 males and females all express genes that affect the outcomes of remating (and P2 fertilization success) because of a likely coevolutionary arms race over the outcomes of these traits (Arnqvist & Rowe, 2005). In an effort to determine whether any of the variation in remating rates among lineages was specifically attributable to genetic variation expressed by P2 males, we ran a separate model, incorporating only data from 'mating class 4' (see Table 1). In this mating class, P2 males of a given nuclear/cytoplasmic lineage competed exclusively against ZA P1 males and ZA females. Although this results in a reduction of the sample size to a quarter of the original sample, and hence the associated statistical power, any significant variation between nuclear/cytoplasmic lineages that is detected must be due to genetic variation expressed by the P2 males.

P2 fertilization success

Cytoplasmic and nuclear effects on the fertilization success of the P2 male (hereafter referred to as 'P2 fertilization success', or simply 'P2') were analysed using a general linear model (type 3), in which the response variable was the proportion of eggs sired by the P2 male, arcsine-transformed. The model was weighted with female fecundity (total egg number on the 14 beans) to account for biases in P2 estimates resulting from cases where fecundity was low (fecundity ranged from 1 to 106, mean: 54.9). The statistical unit of replication was the female. Given that P2 rates are, on average, high in *C. maculatus* (Wilson *et al.*, 1997), with a mean of 73% in this study, females with 0% paternity attributed to the P2 male are unlikely to have been inseminated by the P2 male and were omitted from the analyses to reduce associated error. Females with very low P2 values (i.e. one or two fertile eggs) were included in the analyses. Although some of these females may not have effectively remated (sterility is 99.3% effective), removal of these females only strengthened the statistical significance of the reported patterns. Moreover, repeating the analyses after including all females (including those with 0% P2) or excluding females with fecundity less than 10 eggs resulted in quantitatively similar models in terms of both our ability/inability to reject the null hypotheses and the magnitude of the effects.

The cytoplasmic and nuclear lineage of the P2 male, P1 male type, female type, P1 male age at mating, female age at time of remating (either 24 or 48 h), time of remating (24 or 48 h) and block were entered as fixed factors and the number of eggs on the inducement bean(s) as a fixed covariate. The full model was reduced by excluding non-significant interactions (starting with third order). We employed a bootstrapping resampling procedure, using the residuals of the original model (1000 replicates), to assess the results (ter Braak, 1987; Manly, 1997), because the sample sizes for each group in the model were unbalanced as not all of the females remated. Finally, the results of this model were confirmed using a generalized linear model (with binomial error, logit link, William's correction factor and an identical model reduction protocol as for the remating analysis), which returned a qualitatively identical model. For simplicity, only the final general linear model, with the associated bootstrap probabilities, is presented here. As for the above analysis of female remating, we also analysed mating class 4 separately to determine whether the patterns of variation in P2 among nuclear and cytoplasmic lineages were, in part, attributable to genes expressed by the P2 male.

Results

Remating

Female remating rates varied across the nuclear lineages. Our analysis further showed an interaction between the nuclear lineage and female type for both the whole data set (over 48 h) and the data at 24 h (Table 2). Specifically, there was substantial variation in female remating rates across nuclear lineages when P2 males were exposed to mated females of the same nuclear genotype with which they had coevolved, but little variation when P2 males were exposed to novel ZA females (Fig. 1). This pattern would suggest that most of the nuclear genetic variation underlying this trait is expressed by females, with little influence of P2 male nuclear genes (see Discussion).

The cytoplasmic lineage also had an effect on female remating rates, but this effect was contingent on the P1 male type and only observed at 24 h post-mating (Table 2). The pattern of variation across cytoplasmic lineages differed according to whether P2 males competed against P1 males possessing the same coevolved cyto-type as themselves (controlling for the nuclear background) or against P1 males from the novel ZA stock (ZA nuclear- and cyto-type) (Fig. 2). This cytoplasmic effect was not present in the overall data set (i.e.

Table 2 Effects of nuclear and cytoplasmic genes, P1 male type and female type on female remating rates (a) for the whole data set and (b) at the 24 h exposure.

Source	d.f.	Deviance	*G*	*P*
(a) Remating (whole data set)				
Nuclear lineage	4	71.60	17.90	<0.001
Cytoplasmic lineage	4	2.19	0.55	0.701
Female type	1	5.97	5.97	0.014
P1 type	1	3.30	3.30	0.069
Female age	1	2.78	2.78	0.096
P1 age	1	0.33	0.33	0.565
Block	1	0.18	0.18	0.672
P2 nuclear type × Female type	4	73.02	18.25	<0.001
P2 cytotype × P1 type	4	3.62	0.90	0.461
Total	893	1183.64	1.33	
(b) Remating at 24 h				
Nuclear lineage	4	49.79	12.45	<0.001
Cytoplasmic lineage	4	0.56	0.14	0.968
Female type	1	3.55	3.55	0.060
P1 type	1	1.73	0.67	0.189
Female age	1	1.03	1.03	0.311
P1 age	1	0.42	0.42	0.518
Block	1	0.17	0.17	0.684
P2 nuclear type × female type	4	52.23	13.06	<0.001
P2 cytotype × P1 type	4	13.43	3.36	0.009
Total	893	993.19	1.11	

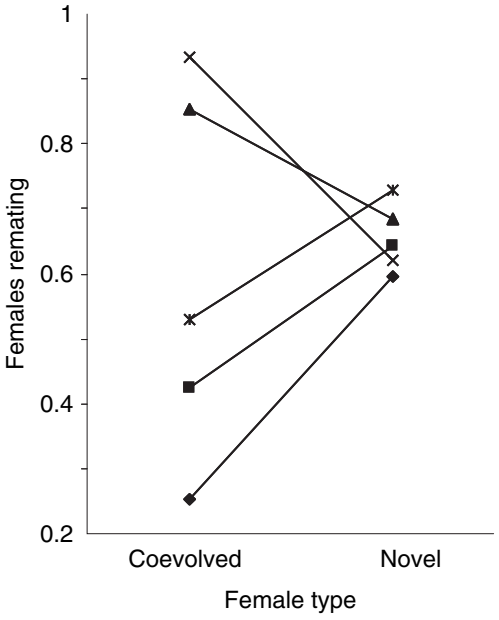

Fig. 1 Proportion of females remating when exposed to P2 males of each nuclear lineage, combining data from 24 and 48 h post-mating. Female type (coevolved vs. novel) is denoted on the horizontal axis, the proportion of females remating on the vertical axis, and each of the five lines represents a distinct nuclear lineage denoted by the following symbols: ◆, BR; ■, CA; ▲, LO; ×, OY; and ✱, YE. Sample sizes for each data point range from 82 to 95.

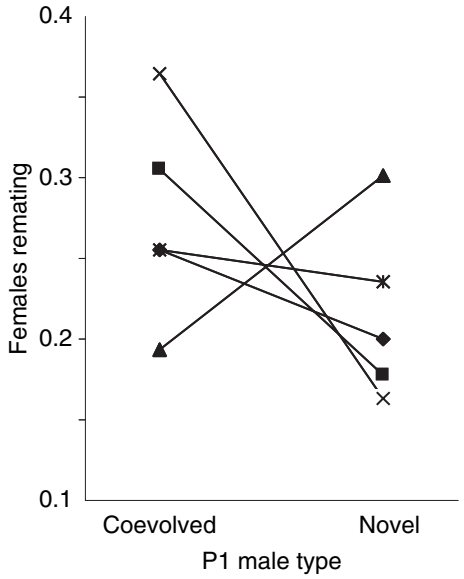

Fig. 2 Proportion of females remating when exposed to P2 males of each cytoplasmic lineage at 24 h post-mating. P1 male type is denoted on the horizontal axis, the proportion of females remating on the vertical axis and each line represents a given P2 cytoplasmic lineage denoted by the following symbols: ◆, BR; ■, CA; ▲, LO; ×, OY; and ✱, YE. Sample sizes for each data point range from 85 to 96.

when combining data from 24 and 48 h exposures) (Table 2).

When we included data only from mating class 4 (ZA P1 males and ZA females), we detected no effects of P2 male nuclear or cytoplasmic genes on remating rates in the overall data set (nuclear: $G = 0.33$, $P = 0.859$; cytoplasmic: $G = 1.78$, $P = 0.129$) or at 24 h (nuclear: $G = 0.40$, $P = 0.806$; cytoplasmic: $G = 1.10$, $P = 0.354$).

P2 fertilization success

There were effects of the nuclear lineage on P2 fertilization success during sperm competition, but the pattern of variation across lineages was contingent on the P1 male type (Table 3, Fig. 3). Furthermore, in four of five nuclear lineages, P2 males clearly had higher fertilization success when competing against P1 males of their own coevolved, rather than the novel ZA genotype (Fig. 3); and this pattern was confirmed by a strong main effect of P1 male type on P2 fertilization success (Table 3). P2 fertilization success was partly contingent on an interaction between the female and P1 male type (Table 3, Fig. 4). Specifically, when P2 males were competing against P1 males with which they had coevolved (i.e. from the same CN line as the P2 male), the female type had little effect on the fertilization success of the P2 male. However, when P2 males were competing with novel (ZA) P1 males, then P2 fertilization success was higher when the female

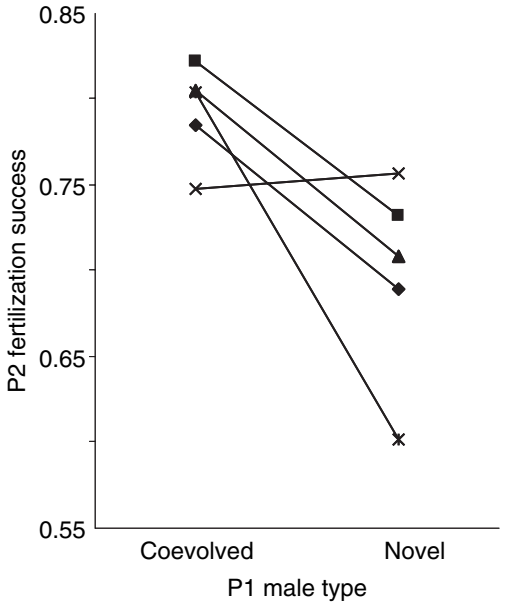

Fig. 3 Mean fertilization success for P2 males of each nuclear lineage when competing against coevolved vs. novel P1 males. P1 male type is denoted on the horizontal axis, P2 fertilization success (back-transformed LS mean values) on the vertical axis and each line represents a given P2 nuclear lineage denoted by the following symbols: ◆, BR; ■, CA; ▲, LO; ×, OY; and ✱, YE. Sample sizes for each data point range from 37 to 77.

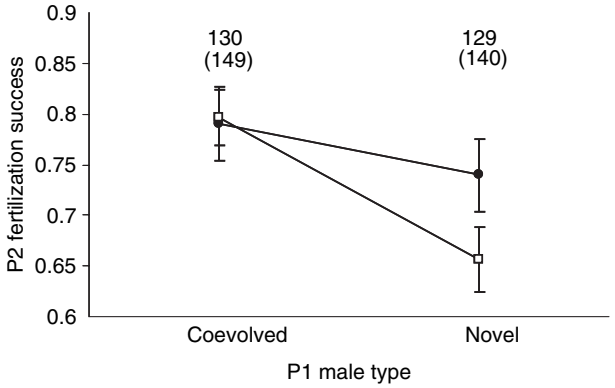

Fig. 4 Fertilization success (back-transformed L.S. means ± S.E.) of P2 males in relation to the P1 competitor male and female type. In any given mating, the P1 male and female were either 'coevolved' (i.e. from the same CN line as the P2 male involved) or 'novel' (i.e. from the ZA stock). P1 male type is denoted on the horizontal axis and P2 fertilization success on the vertical axis. Closed circles denote coevolved females and open squares denote novel (ZA) females. Sample sizes per group are indicated above the bars: numbers above the parentheses indicate sample sizes for rematings involving coevolved females, while numbers within the parentheses indicate rematings involving novel females.

involved was coevolved (i.e. from the same CN line as P2 male) rather than novel (ZA) (Fig. 4). In other words, ZA P1 males had higher fertilization success under sperm competition when mating with ZA females that they had coevolved with.

Table 3 Effects of nuclear and cytoplasmic genes, P1 male type and female type on P2 male fertilization success in sperm competition.

Source	Sum-of-squares	d.f.	*F*-ratio	*P*	*P* (bootstrapped)
P2 nuclear lineage	30.86	4	1.67	0.155	0.136
P2 cytoplasmic lineage	18.20	4	0.99	0.414	0.436
Remating time	13.08	1	2.84	0.093	0.086
Block	30.20	1	6.55	0.011	0.016
Female type	14.30	1	3.10	0.079	0.09
P1 type	75.24	1	16.33	<0.001	<0.001
Female age	33.36	2	3.62	0.027	0.052
P1 age	10.92	1	2.37	0.124	0.114
Egg number on inducement beans	113.33	1	24.59	<0.001	<0.001
P2 nuclear lineage × P1 type	72.83	4	3.95	0.004	0.004
Female type × P1 type	27.97	1	6.07	0.014	0.013
Residual	2423.91	526			

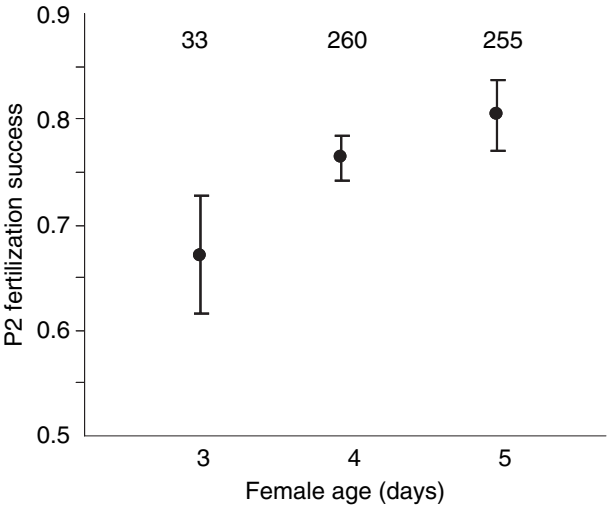

Fig. 5 Mean fertilization success (back-transformed LS mean ± SE) for P2 males remating to mated females of increasing age (in days) since eclosion. Sample sizes are denoted above each group.

P2 fertilization success was also higher when remating to older females (Table 3, Fig. 5). There was also a general effect of block on P2 fertilization success, with higher P2 rates in block 2 (back-transformed LS mean ± SE: block 1 = 0.73 ± 0.02, block 2 = 0.77 ± 0.02). Females with higher fecundity on the inducement beans subsequently laid fewer eggs during the fitness assay ($\beta = -0.50$, $t_{548} = 47.74$, $P < 0.01$) and had lower rates of P2 (Table 3). P2 fertilization success did not vary across the cytoplasmic lineages, and neither were there any interactions between P2 cytoplasmic and nuclear

lineages or cytoplasmic lineage and P1 male or female type on P2.

When we included data only from mating class 4, we detected an effect of nuclear lineage ($F_{4,125} = 4.04$, $P = 0.004$), but not cytoplasmic lineage ($F_{4,125} = 1.14$, $P = 0.400$), on P2 fertilization success. As confirmed by *post hoc* tests (Fisher's LSD: YE vs. OY: $P < 0.001$, vs. LO: $P = 0.002$, vs. CA: $P = 0.006$, vs. BR: $P = 0.087$), this nuclear effect resulted from males of the YE lineage having lower P2 values than males of the other lineages.

Discussion

The experimental design employed here enabled us to disentangle nuclear and cytoplasmic genetic effects on female remating rates and P2 fertilization success in *C. maculatus*. Our results indicate that nuclear genes are the primary genetic contributors to phenotypic variation in both of these traits, and also highlight the importance of interactions between interacting male (P2 and P1) and female characteristics in determining P2 fertilization success. We found that P2 males express nuclear genetic variation for P2 fertilization success. However, P2 males seemingly do not express any such variation for female remating propensity, with the patterns in remating rates across nuclear lineages best explained by genetic variation expressed solely by females. Although we previously identified a cytoplasmic (presumably mitochondrial) genetic component to phenotypic variation in sperm morphology and sperm viability in *C. maculatus* across these same lineages (Dowling *et al.*, 2007a), this cytoplasmic component had no apparent influence on P2 fertilization success. We did, however, detect a seemingly male-mediated cytoplasmic effect on female remating propensity.

Remating

Although this assay was conducted specifically to explore the intergenomic contributions to P2 fertilization success, the design also enabled us to examine these contributions to female remating rates and the results were striking and deserve interpretation. Several studies have now demonstrated that female remating rates may be affected by the genotypes of both the female and competing males (e.g. Van Vianen & Bijlsma, 1993; Sgrò *et al.*, 1998; Andrés & Arnqvist, 2001; Hirano & Miyatake, 2007) and these results have generally been interpreted as evidence for coevolution between the sexes over the outcomes of this trait (Andrés & Arnqvist, 2001; Hirano & Miyatake, 2007).

Here, we found that the female remating rate varied substantially across the five nuclear lineages, but only when P2 males were exposed to female nuclear genotypes that they had coevolved with. In contrast, when P2 males were exposed to mated females of a standardized novel (ZA) genotype, there was little variation in remating rates across nuclear lineages. Furthermore, our analysis of mating class 4 revealed no nuclear genetic variation among P2 males for female remating. Together, these results suggest that the nuclear genes determining female remating rates are expressed primarily by females in *C. maculatus*, which places doubt over whether male offence and female resistance traits for remating rate have coevolved here. We note, however, that the P2 males of each lineage were exposed to only one novel female genotype, which may limit the generality of our conclusion. Furthermore, this assay was not designed to test the genetic effects of the P1 male on female remating, and we suggest that there may well be greater scope for male defensive adaptations, mediated by the P1 male through accessory cell proteins in the ejaculate (see Simmons, 2001; Friberg *et al.*, 2005), to coevolve with female resistance over the outcomes of this trait.

We also found a cytoplasmic genetic component to female remating at 24 h post-mating, which was contingent on the P1 male type. The pattern of variation across cytoplasmic lineages differed according to whether P2 males were competing against P1 males possessing the same cyto-type as themselves or against novel P1 males (ZA nuclear- and cyto-type). This result was surprising as we had little *a priori* reason to expect a cytoplasmic genetic effect on remating rates. Zeh (2004) proposed that the maternal inheritance of cytoplasmic genes sets the stage for the perpetual antagonistic coevolution between nuclear genes involved in male manipulation and cytoplasmic genes involved in female resistance behaviour. Indeed, female remating rate is a trait that is expected to be shaped by strong sexually antagonistic selection (Arnqvist & Rowe, 2005). Unfortunately, our experimental design was not powerful enough to test Zeh's (2004) hypothesis, which explicitly predicts that cytoplasmic genetic variation expressed by females will coevolve antagonistically with nuclear genetic variation expressed in males. However, our results do suggest that the cytoplasmic effects revealed here were mediated through males (both P2 and P1 males) rather than females. Considering the pivotal role of mitochondrial genes in energy production, we suggest that these observed effects may result from each cytoplasmic lineage having a different metabolic capacity. Under this scenario, the males of each cytoplasmic lineage would have different amounts of energy reserves to allocate towards either the harassment of females into remating (in the case of P2 males) or the production of large ejaculates/high sperm numbers (in the case of P1 males, given that females are less likely to remate when receiving a larger ejaculate, Savalli & Fox, 1999).

Regardless of the mechanism underlying this result, these cytoplasmic effects were apparently much smaller than the observed nuclear effects (based on a comparison of effect sizes, i.e. *G*). We also note that these effects were observed only at 24 h post-mating. This is not particularly surprising given that the probability of a female

remating should generally covary positively with the amount of time elapsing since her previous mating. For instance, the females of many species become sperm-depleted over time (Ridley, 1988). Furthermore, in *C. maculatus*, females receive direct benefits such as nutrition (Fox, 1993; Savalli & Fox, 1999; Edvardsson & Canal, 2006; Edvardsson, 2007) from the male ejaculate and the available evidence suggests that these resources are also depleted over time (Savalli & Fox, 1999; Edvardsson, 2007). Thus, it should be easier for a P2 male to convince a mated female to remate at 48 h than at 24 h. By the same logic, it may be easier to detect genetic variation in male offensive adaptations, which are designed to manipulate mated females into remating, earlier in the female 'refractory' period (e.g. 24 rather than 48 h) when a greater proportion of the females tested are still actively resisting remating.

P2 fertilization success

The outcomes of P2 fertilization success were clearly mediated by a combination of P2 male, P1 male and female effects. Thus, P2 fertilization success is not simply a product of the P2 male genotype. Specifically, we found effects of the nuclear lineage on P2 fertilization success, but these effects were dependent on the P1 male type. This interaction resulted primarily from YE P2 males having much lower sperm competitiveness than males of the other lineages when competing against males of the novel P1 genotype. This pattern was confirmed when focusing solely on P2 males that competed against the standardized novel P1 male and female genotype (ZA), and demonstrates that P2 males express nuclear genetic variation for sperm competitiveness, as determined by their ensuing fertilization success, among these lineages. However, we note that YE P2 males had relatively high P2 fertilization success when competing against P1 males (YE) with which they had coevolved. Moreover, P2 males of four of the five nuclear lineages had higher fertilization success when competing with P1 males of familiar, coevolved genotypes than novel P1 males.

These results are consistent with previous experiments that examined male and female contributions to sperm displacement in *D. melanogaster* (Clark, 2002; Bjork *et al.*, 2007) and *C. maculatus* (Wilson *et al.*, 1997). For instance, Clark *et al.* (2000) found that both P2 and P1 male effects, and interactions between the competing males, are important in determining the outcomes of sperm competition in *Drosophila*. In addition to this, they demonstrated that the female genotype (Clark & Begun, 1998) and male × female genetic interactions (Clark *et al.*, 1999) also affect these outcomes. Such results are further supported by Bjork *et al.* (2007), who found that neither sperm offence nor defence responded to experimental selection in an outbred population of *D. melanogaster*, which may possibly reflect a lack of additive genetic variation underlying these traits. They went on to confirm that the sperm competitiveness of a particular male is only repeatable when that male competed against the same rival male for fertilizations within the same female (Bjork *et al.*, 2007). These results are, however, a little surprising as additive genetic variation, albeit at relatively low levels, has been found for both sperm offence and defence in the very same population (Friberg *et al.*, 2005). Wilson *et al.* (1997) have also demonstrated female effects on the outcomes of sperm competition in *C. maculatus*. Specifically, they showed that a given male's sperm competitive success is generally only repeatable when mating with females that are genetically similar (i.e. full sisters). Our study also confirms some female mediation of P2 fertilization success in *C. maculatus*, which by definition invokes a role for cryptic female choice (Eberhard, 1996, 2000). First, we found that an interaction between the P1 male type and female type affected P2 fertilization success. Specifically, P2 males performed very poorly when competing against ZA P1 males for fertilizations inside ZA females. Second, we found that P2 fertilization success was generally higher in older females. This result contrasts with those of Mack *et al.* (2003) who found that P2 fertilization success declines with increasing age in *D. melanogaster*.

It is clear from the above discussion that the sperm competitiveness phenotype of the P2 male is a complex trait that is determined by genetic interactions between the competing males and females. This is an important finding as it shows that this trait is an interacting phenotype (Moore *et al.*, 1997) and that the realized units of selection on the P2 male sperm competitiveness phenotype are pairs or even male–male–female triplets of interacting individuals rather than single individuals. This may have profound effects on the evolutionary trajectory of the sperm competitive phenotype (Moore *et al.*, 1997) and may essentially limit the potential for a short-term directional response in sperm competitiveness by P2 males to the selection imposed by sperm competition. Furthermore, this finding may help to explain the problem of how genetic variation for traits such as sperm competitiveness is maintained. Assuming a minor role for nonadditive gene action, theory states that selection should result in the most competitive alleles for sperm competitiveness becoming fixed within a population (Fisher, 1930; Falconer, 1989; Roff, 1997). However, if the realized units of selection are more commonly pairs (or even triplets) of interacting individuals, rather than single individuals, this can result in frequency-dependent oscillations that facilitate the maintenance of genetic polymorphism (Clark *et al.*, 1999, 2000).

We acknowledge that the results obtained from this study are based on 'between-population' crosses and comparisons (i.e. cyto-types derived from particular populations were introgressed into nuclear backgrounds derived from other populations), similar to those studies conducted by Clark *et al.* (1999, 2000). We used such an approach explicitly to increase the power of finding

genetic effects, as such effects that occur at the 'within-population level' are likely to be more pronounced at the between-population level. Although some of the results found at a between-population level may not hold true at a within-population level, using a between-population approach is an efficient and powerful tool to screen for potential within-population effects. Any such effects should of course then be verified by studies at the within-population level. Notably, the between-population approach also has important merits when it comes to drawing conclusions involving questions related to reproductive isolation and speciation. This is particularly true in the present study given that the evolution of conspecific mating preferences (e.g. Plenderleith *et al.*, 2005; McPeek & Gavrilets, 2006) and conspecific sperm precedence (Howard, 1999; Eady, 2001; Fricke & Arnqvist, 2004) within populations may impose a powerful isolating mechanism when such populations re-establish secondary contact.

It is now established that, at least in some taxa (e.g. humans and beetles), a cytoplasmic genetic component underlies phenotypic variation in several sperm traits presumed to be important in sperm competition (Ruiz-Pesini *et al.*, 2000; Dowling *et al.*, 2007a). In an earlier study, we reported cytoplasmic genetic contributions to two sperm traits, viability and length, in *C. maculatus* (Dowling *et al.*, 2007a). This cytoplasmic component is most likely attributable to the presence of mitochondrial genetic polymorphism across the cytoplasmic lineages used given that: (i) we eliminated the possibility of bacterial infection among the lineages by treating them with antibiotics, and (ii) there is abundant mtDNA polymorphism among laboratory strains of *C. maculatus* (G. Arnqvist and J. Rönn, unpubl. data; Dowling *et al.*, 2007b). Given these findings, the primary aim of this study was to test whether variation in cytoplasmic genes affects the actual outcomes of sperm competition, in particular the fertilization success of the P2 male. Such effects, depending on their magnitude, could limit the scope for the sperm competitiveness phenotype to evolve adaptively. We were, however, unable to detect a cytoplasmic effect on P2 fertilization success. Based on our results, it thus appears unlikely, at least in *C. maculatus*, that cytoplasmic genes will impose significant genetic constraints on the adaptive evolution of sperm competitiveness.

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Supplementary Material

The following supplementary material is available for this article:

Figure S1 The efficiency of the male sterility protocol with time since irradiation.

This material is available as part of the online article from: http://www.blackwell-synergy.com/doi/jeb/10.1111/j.1420-9101.2007.01433.x

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