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Applying the genetic theories of ageing to the cytoplasm: cytoplasmic genetic covariation for fitness and lifespan

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mutation accumulation;
senescence.

Abstract

Two genetic models exist to explain the evolution of ageing – mutation accumulation (MA) and antagonistic pleiotropy (AP). Under MA, a reduced intensity of selection with age results in accumulation of late-acting deleterious mutations. Under AP, late-acting deleterious mutations accumulate because they confer beneficial effects early in life. Recent studies suggest that the mitochondrial genome is a major player in ageing. It therefore seems plausible that the MA and AP models will be relevant to genomes within the cytoplasm. This possibility has not been considered previously. We explore whether patterns of covariation between fitness and ageing across 25 cytoplasmic lines, sampled from a population of *Drosophila melanogaster*, are consistent with the genetic associations predicted under MA or AP. We find negative covariation for fitness and the rate of ageing, and positive covariation for fitness and lifespan. Notably, the direction of these associations is opposite to that typically predicted under AP.

Introduction

The genetic models of the evolution of ageing are grounded on the logic that the force of natural selection decreases with age (Medawar, 1952; Williams, 1957; Hamilton, 1966). Two alternative models have been proposed. The first proposes that deleterious mutations that are expressed predominantly in late-life [or rather, mutations with deleterious effects that manifest themselves only at a late age (Partridge & Barton, 1993) or escalate in their effect with age (Houle *et al.*, 1994)] accumulate due to the declining, age-dependent force of selection. This is the mutation accumulation (MA) theory of ageing (Medawar, 1952). The second model proposes that mutations with deleterious effects in late-life can accumulate when they simultaneously confer benefits early in life. These alleles are thus selected despite their negative (antagonistic) pleiotropic effects on late-life performance (Williams, 1957). This is the antagonistic pleiotropy (AP) theory of ageing. These models have been tested extensively and empirical evidence has been found for each (e.g. Hughes *et al.*, 2002; Snoke & Promislow, 2003). Although the relative importance of AP vs. MA to ageing still remains debated (Charlesworth, 2001; Partridge & Gems, 2002; Snoke & Promislow, 2003; Hughes & Reynolds, 2005), there have been recent suggestions that the weight of evidence (primarily the results of experiments utilizing artificial selection in *Drosophila*) favours AP as the more significant player (Partridge & Gems, 2002; Hughes & Reynolds, 2005).

One useful indicator that enables a comparison of the likely contribution of MA in relation to AP to genetic variation for ageing is provided by the direction of the genetic association between early-life fitness and ageing. Under AP, early-life fitness and the rate of ageing are typically predicted to be positively (and early-life fitness and lifespan negatively) related, given that mutations that are beneficial and hence positively selected in early life are detrimental in later life (Rose & Charlesworth, 1981; Hughes, 1995; Zwaan, 1999; Snoke & Promislow, 2003). The expected association under MA is more

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complicated. Under a traditional model of MA, the effects of alleles that cause ageing will be confined to a single age class. Under this scenario, the mutations that accumulate and causing ageing will mostly have deleterious effects late in life, but no effects on earlier life stages, and therefore there should be no association between early-life fitness and ageing. However, a modified model of MA has recently been discussed, in which alleles causing ageing may have effects spanning over more than one age class (Charlesworth, 2001; Reynolds *et al.*, 2007). Under this scenario, mutations with late-life effects will be correlated with weak (but nonetheless detectable) deleterious effects on fitness in earlier life stages (i.e. they are not entirely neutral to selection). If so, then increases in the number of late-acting deleterious mutations that accumulate under mutation-selection balance will both speed up the rate of ageing and depress fitness (Tatar *et al.*, 1996; Charlesworth, 2001; Reynolds *et al.*, 2007), and a negative relationship between fitness and the rate of ageing (and a positive relationship between early-life fitness and lifespan) could exist across genotypes. Thus, in summary, when MA is the chief cause of ageing, early-life fitness could either be uncorrelated with, or negatively related to, the rate of ageing (and positively related to lifespan) across genotypes.

Mitochondrial DNA (mtDNA) represents the core extra-nuclear component of DNA within the cytoplasm of animal cells [although a range of bacteria, such as *Wolbachia* (Clark *et al.*, 2005), and possibly certain viruses and protists (Clark, 1985; MacRae & Anderson, 1988; Dowling *et al.*, 2008) also contribute to the total pool of cytoplasmic DNA in many species]. Although controversial (Khrapko *et al.*, 2006; Khrapko & Vijg, 2007), the idea that mtDNA plays a causal role in the ageing process is well established (e.g. Brand, 2000; Mandavilli *et al.*, 2002; Ballard & Whitlock, 2004) and several lines of evidence exist to support this assertion. First, damage to mtDNA and decreases in mitochondrial enzymatic activity are known to increase with age (Sohal & Weindruch, 1996; Wallace, 1997; Yan *et al.*, 1997; Schwarze *et al.*, 1998; Ferguson *et al.*, 2005). Second, several studies have revealed associations between naturally occurring levels of mitochondrial or cytoplasmic genetic variation and patterns of ageing in *Drosophila* (James & Ballard, 2003; Maklakov *et al.*, 2006; Rand *et al.*, 2006; Clancy, 2008). Third, two recent studies using transgenic mouse models (mtDNA mutator mice), which exhibit an increased rate of somatic point mutations in mtDNA, found seemingly causal effects of mtDNA mutations on ageing (Trifunovic *et al.*, 2004; Kujoth *et al.*, 2005). However, it is worth noting that the results of these two studies were recently disputed by Vermulst *et al.* (2007), who found that these transgenic mice were able to sustain a 500-fold higher mutation burden than wild-type mice without any indications of accelerated ageing.

Nonetheless, considering that the conceptual link between mtDNA and ageing is so strong (e.g. Harman, 1972; Lambert & Brand, 2007), and that evidence is emerging to suggest that naturally occurring cytoplasmic (presumably mitochondrial) genetic variation within populations is linked to variance in ageing (Maklakov *et al.*, 2006), it seems striking that nobody has previously posed the question of whether cytoplasmic genetic variation may accumulate according to a pattern that is consistent with either the MA or AP genetic models of the evolution of ageing. Indeed, such a test is particularly timely in the light of the recent studies that have presented evidence that mtDNA variation within populations is shaped by natural selection (e.g. reviewed in Rand, 2001; Ballard & Whitlock, 2004; Meiklejohn *et al.*, 2007; Dowling *et al.*, 2008). Thus, the force of selection acting on mtDNA mutations will decrease with age and mutations may conceivably accumulate according to either of these models. Furthermore, it is reasonable to expect that deleterious mutations will equilibrate within the mitochondrial genome under mutation-selection balance. For, on the one hand, we would expect selection to rapidly purge or fix any non-neutral genetic variation given that it is a haploid genome with no opportunity for heterozygosity to mask non-neutral mutations from selection. But, on the other hand, the efficacy of selection acting on mtDNA is generally thought to be lower than on nuclear DNA, given that the effective population size (N_e) of the mitochondrial genome is likely to be lower than that of its nuclear counterpart (but see Ballard & Whitlock, 2004 who discuss conditions under which this difference in N_e among nuclear and organelle markers might not apply). Any relative reduction in N_e of the mitochondrial genome is largely attributable to its generally strict maternal transmission (Lynch, 1997), acknowledging that a small amount of paternal leakage of mtDNA probably occurs in some species (e.g. Kondo *et al.*, 1990). Furthermore, the N_e of the mitochondrial genome will be further reduced through a number of molecular genetic effects (known as Hill–Robertson effects) that are pertinent to nonrecombining haploid genomes, e.g. Muller's ratchet, background selection and genetic hitchhiking (Hill & Robertson, 1966; Berlin *et al.*, 2007; but see Tsaousis *et al.*, 2005 for evidence suggesting signals of recombination in animal mtDNA).

Here, and for the first time, we ask the question whether cytoplasmic genetic variation may accumulate in a pattern consistent with either AP or MA. We use data collated from two published studies that demonstrated cytoplasmic genetic variation for female fitness (Dowling *et al.*, 2007) and female ageing (Maklakov *et al.*, 2006) within the same population of *Drosophila melanogaster*, to test for genetic associations between fitness and lifespan/patterns of ageing across 25 cytoplasmic lines (cytolines, possessing controlled nuclear backgrounds) and two confirmed mtDNA types (based on sequencing 14% of the total amount of mtDNA – see Materials and methods). We discuss our findings in relation to the patterns predicted under the different genetic models of

ageing, and consider the potential consequences of this idea for our general understanding of the evolution of ageing.

Materials and methods

Laboratory flies and creation of 'cyto' lines

In brief, the flies used in this analysis were sampled from a large, outbred laboratory population (LH_M - abbreviation for Larry Harshman, moderate density population) of *D. melanogaster*, originally founded by 400 mated females in 1991, collected by Lawrence G. Harshman (University of Nebraska, USA), and subsequently maintained by William R. Rice (University of California, Santa Barbara, USA) under controlled laboratory conditions at a population size of 1800 breeding adults. LH_M is cultured on a 14-day discrete generation cycle and has adapted to the laboratory environment for over 300 generations (see Rice *et al.*, 2005 for details of the culturing protocol for this population).

Cytoplasms were sampled from the LH_M base population by randomly collecting 25 mated females. Each of these females was then used to found a separate 'cyto'-line, fixed for her cyto-/mito-type. These lines are referred to as *mt lines* in Maklakov *et al.* (2006) and Dowling *et al.* (2007). A protocol of introgressive backcrossing was used to disentangle each sampled cytoplasm from the nuclear background with which it was originally associated. Specifically, daughters from each line were backcrossed to randomly sampled males from LH_M over 27 generations. Although initially these backcrosses used only small numbers of flies (eight daughters per cyto-line backcrossed to eight random males from LH_M for the first 20 generations), sample sizes were increased in later generations to ensure that sampling error would not create differences in the nuclear genetic backgrounds represented within different cyto-lines. Specifically, in generations 21–25, the number of daughters used for each backcross was increased to an average of 70 (range: 42–100) and the number of random base males from LH_M to 50. At generation 26, each line was further increased to contain 150 daughters and 100 random LH_M males, distributed equally across five vials per cyto-line. All lines were treated with antibiotics in generation 26 to eliminate any potential intracellular bacterial infections, such as *Wolbachia*. This protocol resulted in an estimated 99.92% of the original nuclear background of each cyto-line being replaced by generation 27, with a large random sample of alleles representative of LH_M. Given that the later seven generations of backcrossing involved large numbers of males from LH_M, we expect that there were no differences in the representation of nuclear genetic background between each of the cyto-lines. From generation 28, the lines were closed and each subsequent generation was propagated in five replicate vials per cyto-line, by 32 pairs/vial, with complete admixture of adults between vials at the commencement of each new generation. Subsequent egg densities were counted and trimmed to 150–200 eggs/vial. See Dowling *et al.* (2007) for specific details of this protocol.

mtDNA variation

Four protein-coding mtDNA gene fragments (encompassing a total of 2752 base pairs of mtDNA) were sequenced in each of these 25 cyto-lines. These fragments were *CytB*, *Cox2* and two nonoverlapping fragments within *ND5*, and represent about 14% of the total amount of mtDNA of *D. melanogaster* (see Dowling *et al.*, 2007). This sequencing effort revealed a single nonsynonymous nucleotide polymorphism in *CytB* (site 204 showed a transition – A in cyto-lines 6 and 11, G in the remaining 23 lines, corresponding to an amino acid replacement: *Tyr* in lines 6 and 11, *Cys* in the other lines). Thus, there are at least two confirmed mtDNA variants among the 25 cyto-lines, with further hidden polymorphism likely given that we only sequenced about 14% of the mitochondrial genome.

Definitions of female fitness and ageing parameters

The fitness assay was initiated three generations after the conclusion of the backcrossing protocol, and continued over three sequential experimental blocks. To commence the assay, 4-day-old females of each cyto-line were mated to randomly-sampled 3-day-old males from the LH_M base population for 2 h (females will mate once during this time period; Rice, 1996; Holland & Rice, 1999). Each female was then provided with her own vial, containing a prescribed amount of live yeast (0.8 mg), in which she resided for 18 h before being discarded. The number of offspring emerging from these vials was scored 11 days later. This measure is relatively closely related to total adult female fitness in this population, as defined by the culturing protocol these flies have been adapting to for over 300 generations. However, when viewed from an ageing perspective, it is appropriate to regard this measure as one of early-life female fitness, given that these flies have yet not fully adapted to their new life history in the laboratory. This observation is evident by (a) their long post-reproductive lifespan (see below) and also (b) the observation that the amount of live yeast provided to females must be periodically reduced in order to control egg production at a constant rate over time (Long *et al.*, 2009).

On average, 180 females were assayed per cyto-line. The fitness of each cyto-line was assessed across three controlled haploid nuclear backgrounds (i.e. 60 females assayed per haploid background per cyto-line) that were drawn from the LH_M population, whereas the other haploid component was randomly drawn (per assayed female) from the same population. Thus, the fitness of each given cyto-line here encompasses the mean fitness

when expressed across these three haploid nuclear backgrounds. Further details of the fitness assay are described in Dowling *et al.* (2007).

Ageing parameters were calculated by scoring the mortality of 250 mated females from each cyto-line. The assay was conducted in the fourth generation following the backcrossing protocol. These females had been previously exposed and thus mated, as virgins and in groups of 28, to randomly selected males from the LH_M base population for 2 h. Females of each line were then distributed evenly across five replicates (50 females per vial) to commence the assay (Maklakov *et al.*, 2006). Each replicate of females was transferred to a new vial, containing an *ad libitum* amount of live yeast, every second day and the number of dead flies recorded. Mean lifespan (days) per line was then calculated, and rates of senescence calculated in WinModest 1.0.2 (Pletcher, 1999). The mortality function in the data set was best described by the Gompertz model $\mu_x = \alpha e^{\beta x}$, where μ_x is the predicted instantaneous mortality rate at age x, α is the Gompertz intercept or *baseline mortality rate* (referred to as frailty in Maklakov *et al.*, 2006) and β is the rate of increase in mortality with age or the *rate-of-senescence* of the population (Finch, 1990; Pletcher, 1999). The ageing parameters per cyto-line denote mean values across five vials, with each cyto-line expressed alongside a random diploid nuclear background, again presuming the nuclear allelic constitution of each cyto-line matched that segregating within LH_M as a whole. See Maklakov *et al.* (2006) for further details.

Statistical rationale and background

By treating the cyto-lines as random effects in restricted maximum likelihood models, Dowling *et al.* (2007) and Maklakov *et al.* (2006) addressed the question of whether within-population cytoplasmic genetic variation exists, in general, for fitness and ageing in *D. melanogaster*. They were also able to estimate the amount of variance in these traits explained by such genetic variation.

Here, we test for an association between mean fitness and ageing parameters (baseline mortality rate, rate-of-senescence and lifespan,) across the 25 cyto-lines, using linear regression. By doing so, we directly address whether cytoplasmic genetic variation for ageing and fitness is consistent with the MA or AP models of ageing. Although our results do not hinge on the assumption, it seems reasonable to assume that any cytoplasmic genetic variation detected for these two traits might reflect underlying variation in mtDNA among the cyto-lines, given that we treated all lines with antibiotics to eliminate cytoplasmically transmitted bacteria such as *Wolbachia* (see Discussion). Nonetheless, we also take the further step of testing for differences in fitness and ageing parameters across the two confirmed mtDNA variants (using *t*-tests) and visually examining the pattern of covariation between these variables. The unit of replication for these *t*-tests was the cyto-line means (23 of the lines were characterized by one mtDNA variant and the other two lines by the rare variant).

Results

Fitness and ageing among cyto-lines

There was a positive association (but not significant at alpha = 0.05) between mean fitness and lifespan across the 25 cyto-lines ($\beta = 0.15$, $t_{24} = 2.0$, $P = 0.058$, $r^2 = 0.15$). Visual inspection of the relationship between these two variables revealed that one particular cyto-line (line 7) deviated from the mean lifespan by more than four standard deviations (Fig. 1a). This line was a statistical outlier in the model, having a large Studentized Residual (3.16) and large leverage (0.71). Following the removal of this line, the relationship between the two parameters was highly significant ($\beta = 0.46$, $t_{23} = 3.93$, $P < 0.001$, $r^2 = 0.41$).

The relationships between mean fitness and baseline mortality (Including line 7: $\beta = -2.20$, $t_{24} = -0.43$, $P = 0.67$, $r^2 = 0.01$; Excluding line 7: $\beta = -0.15$, $t_{23} = -0.82$, $P = 0.42$, $r^2 = 0.03$), and fitness and rate-of-senescence (Including line 7: $\beta = 1.22$, $t_{24} = 0.13$,

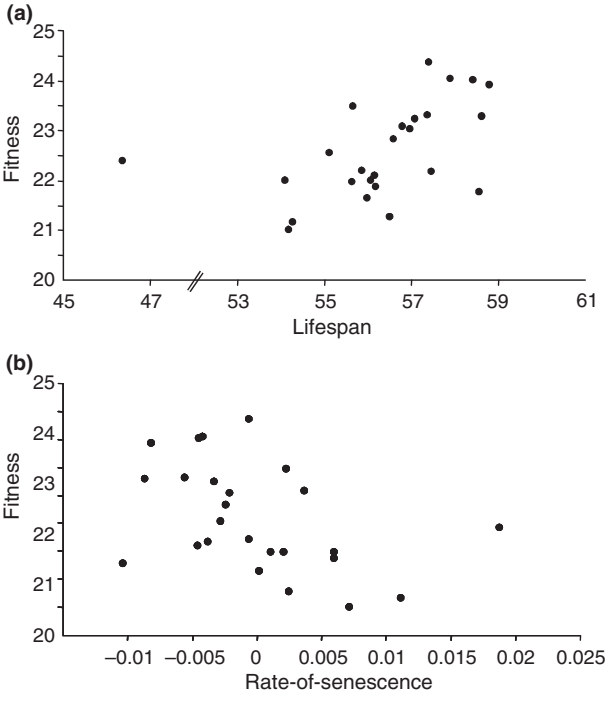

Fig. 1 Mean female fitness (number of offspring) vs. (a) lifespan (days) and (b) Gompertz rate-of-senescence (controlled for baseline mortality, ln α) for the 25 cyto-lines. Note that the scale for lifespan is broken (between 47 and 53) to show the position of the outlier (line 7). The outlying data point to the right in (b) also represents line 7.

$P = 0.90$, $r^2 = 0.00$; Excluding line 7: $\beta = 0.81$, $t_{23} = 0.08$, $P = 0.93$, $r^2 = 0.00$), were not significant in isolation. However, the baseline mortality (α) and the rate-of-senescence (β) are generally negatively phenotypically correlated (Hughes, 1995), and such a correlation was confirmed in our data set (Maklakov *et al.*, 2006). When variation in the rate-of-senescence due to baseline mortality was controlled for (by taking the residuals of the rate-of-senescence on baseline mortality, *sensu* Miyo & Charlesworth, 2004), there was a negative association between fitness and the rate-of-senescence (Including line 7: $\beta = -64.64$, $t_{24} = -2.36$, $P = 0.027$, $r^2 = 0.19$; Excluding line 7: $\beta = -134.24$, $t_{23} = -4.13$, $P < 0.001$, $r^2 = 0.44$; Fig. 1b) across the 25 lines. That is, lines that had relatively higher fitness aged slower than lines with relatively lower fitness.

Fitness and ageing of mtDNA variants

We then checked whether the mtDNA polymorphism that we detected among the cyto-lines (confirmed by the sequencing of 14% of the mtDNA genome) was related to the patterns of variation in fitness and ageing among cyto-lines. When using standard pooled-variance *t*-tests, the rate-of-senescence (controlling for baseline mortality) differed between the two mtDNA types, at least when line 7 was excluded from the analysis (Including line 7: $t_{23} = 2.02$, $P = 0.055$; Excluding line 7: $t_{22} = 2.31$, $P = 0.030$). Differences in lifespan were only detected when the outlying line 7 was excluded from the analysis (Including line 7: $t_{23} = -1.58$, $P = 0.127$; Excluding line 7: $t_{22} = -2.56$, $P = 0.018$), whereas fitness did not differ across the two mtDNA types (Including line 7: $t_{23} = -1.63$, $P = 0.118$; Excluding line 7: $t_{22} = -1.63$, $P = 0.128$). However, the variances for each of these variables differed substantially between the two mtDNA types (by more than 300 times in the case of lifespan and rate-of-senescence), and Bartlett's tests of homogeneity of variance indicated that variances were heterogeneous (lifespan: $F = 322.46$, $P = 0.088$; rate-of-senescence: $F = 370.25$, $P = 0.082$), noting that the power of these tests was small given that one group contained only two observations.

Given the unequal variances, we reanalysed the data using Behren–Fisher (BF) *t*-tests with Monte Carlo simulations (based on 50 000 iterations) to estimate more precise *P*-values. This test is not only more appropriate when analysing small data sets with unequal variances between groups, but is also considered relatively conservative (Barnard, 1984). The BF *t*-tests revealed that both lifespan (Including line 7: $P = 0.023$; Excluding line 7: $P = 0.026$) and the rate-of-senescence (Including line 7: $P = 0.016$; Excluding line 7: $P = 0.018$) differed between the two mtDNA types, whereas fitness did not (Including line 7: $P = 0.185$; Excluding line 7: $P = 0.186$). The lack of statistical significance for fitness is quite likely attributable to low statistical power given that the rarer mtDNA type contained only two data points. However, we note that if the means of each of these variables are plotted, a positive genetic association is apparent between fitness and lifespan, and a negative association between fitness and the rate-of-senescence, across the two mtDNA types (Fig. 2). In short, if any true association does exist between mean fitness and the mean of these ageing parameters across the two mtDNA types, then it most likely occurs in the same directions as we observed across all cyto-lines.

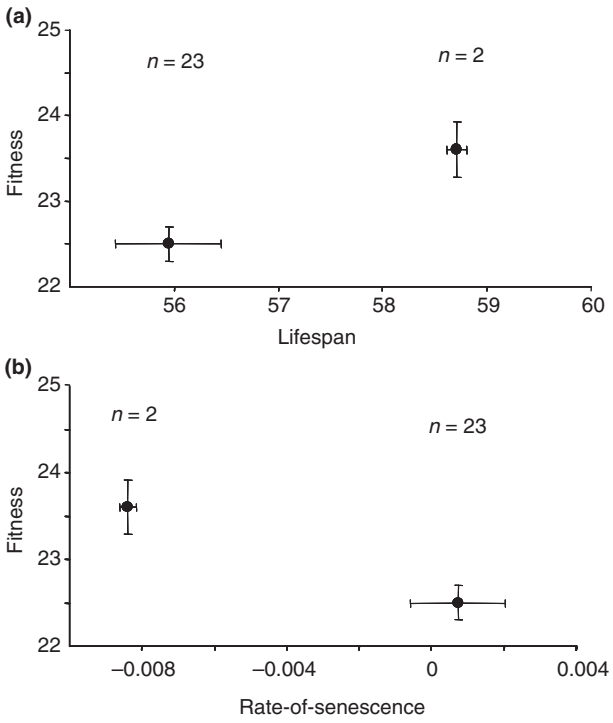

Fig. 2 Mean (± 1 SE) female fitness (number of offspring) vs. (a) mean female lifespan (days) and (b) mean female Gompertz rate-of-senescence, β (controlled for baseline mortality, ln α) for the two mtDNA types. Sample sizes (i.e. number of cyto-lines) are denoted above each data point.

Discussion

We found a positive genetic association between fitness and lifespan, and a negative genetic association between fitness and the rate of ageing, over the 25 cyto-lines studied. This pattern of covariation is consistent with a modified model of MA in which mutations with late-life deleterious effects confer weak deleterious effects on fitness at earlier life stages. What is most striking, however, is that this pattern is directly opposite to that predicted under AP. Furthermore, the direction of the associations found across the cyto-lines was consistent with the direction observed across the two confirmed mtDNA types, which supports our interpretation that

these reported correlations represent true biological phenomena. We do, however, acknowledge that the difference in mean fitness across the two mtDNA types was not statistically significant, which may well reflect the limited statistical power of these tests (see Results).

A modified model of MA, in which mutations that affect ageing exhibit moderate (rather than strict) age-specificity, has received recent theoretical attention (Charlesworth, 2001), and such a model is backed up by some empirical support. For instance, Reynolds *et al.* (2007) assessed the degree of age-specificity of segregating alleles affecting fitness in a population of *D. melanogaster* and found positive genetic correlations extending over 1–2 weeks (with weak correlations spanning 3 weeks), across all age classes, thus supporting the model. These findings are consistent with several others that found evidence for the existence of spontaneous mutations (Houle *et al.*, 1994; Pletcher *et al.*, 1998, 1999) or segregating alleles (Curtsinger & Khazaeli, 2002) with positively correlated effects across age-classes.

An alternative explanation for the genetic correlations we found between early-life fitness and ageing parameters is that they reflect segregating beneficial alleles, with positive effects that are expressed across age-classes. Although we cannot rule out this possibility, we note that the frequency of deleterious mutations is normally assumed to be much higher than the frequency of beneficial mutations (Houle *et al.*, 1994), and this would seem to hold true for mutations in mtDNA (Rand, 2001). Furthermore, we note that the LH_M population is still adapting to an early fitness life history regime (see Materials and methods). Specifically, flies are discarded after their fifth day of adulthood and only eggs oviposited in the 18 h preceding this are used to propagate the subsequent generation. Under this new laboratory-imposed regime, mutations with beneficial effects that are expressed in the first 5 days of adulthood will be favoured regardless of the magnitude of any deleterious pleiotropic effects that they have later in life. To this end, the move from the wild to the laboratory life-history regime will have resulted in a shift in the subset of mutations, within the total mutation distribution, that are treated as beneficial. That is, mutations that might have been under purifying selection in the wild (if associated with adverse effects expressed at life-stages after day 5) will now be under positive selection if they confer any advantages within the first 5 days of adulthood. Thus, in the new laboratory environment, a greater proportion of the total pool of beneficial alleles, which are sweeping through the population, will have antagonistic pleiotropic effects as the population adapts to its new life-history regime. Thus, if beneficial alleles segregating in the population were responsible for driving the patterns of covariance, then it seems likely that this would have resulted in the opposite pattern of covariance (in line with AP) to that which we observed.

Possible confounding effects?

Previously, several authors have noted that spurious positive correlations between fitness and lifespan may arise if the population under examination is inbred (Rose & Charlesworth, 1981; Rose, 1984; Clark, 1987), subjected to novel environmental conditions (Service & Rose, 1985; Clark, 1987) or if the correlations presented are phenotypic rather than genetic (Rose, 1991). Our results are unlikely to suffer from any of these problems. First, the correlations presented are genotypic (fitness and mortality were measured in different assays with different flies, and the assayed flies of each cyto-line were derived from many sets of outbred parents and sampled over multiple vials). Second, the flies used were not inbred and each experiment was conducted under highly standardized environmental conditions, and using a population that has been adapting to this laboratory environment for over 300 generations (Dowling *et al.*, 2007).

It is worth considering whether the associations reported here could result from any nonrandom, or nonstandardized, variation in the nuclear background across the cyto-lines. This possibility is unlikely given that the backcrossing protocol was extensive and involved matings to large numbers of males from LH_M in the later generations (e.g. 150 daughters per line backcrossed to 100 randomly sampled males from LH_M in the later generations). Thus, at the conclusion of backcrossing, it seems improbable that there would be any differences in the pool of nuclear alleles, or their relative frequencies, between any of the cyto-lines. Furthermore, sample sizes in both assays were high (mean fitness per cyto-line was estimated from a total of 180, and ageing parameters from a total of 250, females per line), and thus it is unlikely that we captured a biased sample of the segregating nuclear alleles within each cyto-line. Finally, given that the fitness and ageing assays commenced soon after the conclusion of the backcrossing protocol (within four generations), it seems unlikely that the nuclear genetic backgrounds associated with any of the cyto-lines had sufficient time to become distinct, through drift or selection. We concede that it is possible that selection on the cyto-nuclear genotype could, for example, favour certain nuclear allelic variants in particular cyto-lines over others, but such selection would need to be strong to result in detectable differences in the nuclear background between cyto-lines by the commencement of the assays.

In the fitness assay, the cyto-type of each female was screened against one of three standardized haploid nuclear backgrounds, with the other haploid component randomly drawn from the pool of haploid nuclear backgrounds within each cyto-line, which presumably matched the total available pool of haploid nuclear backgrounds found in the LH_M population. Mean fitness per cyto-line thus reflects the estimated fitness of each

cyto-type when averaged across three standardized haploid nuclear backgrounds drawn from LH_M. In the ageing assay, the cyto-type of each female was screened against a diploid component of random nuclear alleles drawn from a pool of alleles that reflected the total available pool in LH_M. The mean ageing parameters per cyto-line thus reflect the estimated values when averaging across all possible nuclear backgrounds within LH_M. Could these assay-specific differences in the genetic background be responsible for driving a spurious pattern of covariation between fitness and ageing? We do not think so. One limitation of the fitness assay is that we only measured fitness in three haploid backgrounds, and therefore the nuclear genetic variation in fitness that we observed might not be representative of the fitness variation present when measured across all possible backgrounds. We also acknowledge that there is scope for other aspects of the genetic background of the focal females to have differed between the fitness and ageing assays. For instance, there was possibly a lower frequency of expression of deleterious recessive alleles within the nuclear backgrounds of focal females in the fitness assays, as such alleles were likely to have been purged when creating the inbred lines that contributed to their standardized haploid backgrounds (see Dowling *et al.*, 2007). However, it does not seem likely that such differences discussed here could affect the pattern of covariation seen across cyto-lines for fitness and ageing. The important point is that within each of these assays, there was little opportunity for nonrandom differences in nuclear genetic variation to exist between the cyto-lines, and therefore the most likely explanation for this pattern of covariation is that it reflects genetic variation within the cytoplasm.

This study explores whether cytoplasmic genetic covariation exists for fitness and ageing, but we acknowledge that there is an underlying assumption that the cytoplasmic variation detected most likely reflects mitochondrial genetic variation. How strong is the evidence to support such an assumption? A range of cytoplasmically transmitted bacteria, such as *Wolbachia*, are common in *Drosophila* (Clark *et al.*, 2005), and given that these micro-organisms are assumed to be in near-perfect linkage with the mitochondrial genome, it is difficult to disentangle their effects from those of the mitochondrial genome. In this study, all cyto-lines were treated with antibiotics at least six generations prior to the assays commencing to eliminate the possibility that cytoplasmically transmitted bacteria, such as *Wolbachia*, would confound the results of this study. The observed results may also be caused or confounded by variation among cyto-lines in infection with a range of cytoplasmically transmitted viruses (Clark, 1985; MacRae & Anderson, 1988; Dowling *et al.*, 2008), the prevalence of which are unknown in our population. However, the available evidence suggests that the fitness effects attributable to such viruses are minimal (Shabalina *et al.*, 1997). Finally, given that the direction of the reported patterns between fitness and ageing (albeit with a nonsignificant difference in fitness) among the two confirmed mtDNA variants (based on sequencing 14% of the total amount of mtDNA) was the same as that among the 25 cyto-lines, we argue that the best explanation for the associations reported here is that they reflect variation in mtDNA among lines.

Theoretical considerations

The genetic correlations observed here were in the opposite direction to those typically predicted by the AP model of ageing (e.g. Hughes *et al.*, 2002; Snoke & Promislow, 2003). Some might argue that it is not surprising that we failed to support the AP model, given that it is not immediately obvious how standing genetic variance could be maintained within the mitochondrial genome by AP. Traditionally, balancing selection via heterozygote advantage was considered a plausible mechanism via which antagonistic pleiotropic mutations could be maintained within populations (Rose, 1982, 1985). However, the general importance of AP in the maintenance of genetic variation has since been questioned (see Curtsinger *et al.*, 1994; Hedrick, 1999). At any rate, there is no scope for heterozygote advantage to maintain genetic variation within the cytoplasmic genome given it is haploid (hence no heterozygosity).

Other theoreticians have tackled the question of whether selection may contribute to the maintenance of genetic polymorphism within the cytoplasm, and they concluded that some forms of balancing selection, such as frequency-dependent selection (Gregorius & Ross, 1984), or sexually antagonistic selection on cyto-nuclear genetic combinations (Rand *et al.*, 2001), might contribute to upholding this variation. Indeed, recent empirical work suggests that sex-specific cyto-nuclear fitness interactions may be more common than previously thought (Rand *et al.*, 2001; Dowling *et al.*, 2007; Wayne *et al.*, 2007). Thus, the possibility may exist for antagonistic pleiotropic mutations within the cytoplasm to be maintained by one of these latter processes. Nonetheless, on the balance of the available theory, it seems that the haploidy of cytoplasmic genomes might predestine them to accumulate more genetic variation in ageing due to MA than AP, in comparison with diploid nuclear genomes where other forms of balancing selection can operate more freely. This is also in line with our results, which suggest that the accumulation of mutations with antagonistic pleiotropic effects within the cytoplasmic genome appears to be unimportant in the evolution of ageing, at least in this population. Of course, this conclusion only pertains to the standing genetic variation present across the cyto-lines in this study, and does not concern alleles with effects on ageing that have already become fixed within the cytoplasmic genome. Furthermore, we were searching for relationships between cytoplasmically encoded

fitness and ageing parameters over whole cytoplasmic/mitochondrial haplotypes, which of course does not preclude the possibility that some individual genes within the cytoplasm may accumulate variation according to the AP model (see Leroi *et al.*, 2005).

As stated previously, the LH_M population used here is cultured in a way that promotes the likelihood of late-acting deleterious mutations (expressed after day 5 of adulthood) accumulating in the nuclear and mitochondrial genomes (Medawar, 1952; for a detailed discussion of such effects in laboratory cultures of *Drosophila*, see Promislow & Tatar, 1998). Given that selection is acting exclusively early in life in this population, with an abrupt decline in selection after day 5, genetic variation for traits expressed late in life, such as ageing, is likely to be elevated here in relation to that in natural populations. This presumably increased our power to detect associations between fitness and patterns of ageing among the cyto-lines and mtDNA types in this study. It is, however, difficult to predict whether this culturing protocol would have played any role in driving the sign of the associations between fitness and ageing across cyto-lines in this study. On the one hand, this culturing protocol essentially mimics a MA experiment, as alleles with effects expressed after day 5 of adulthood will escape direct selection. This will therefore presumably have increased the chances of us finding support for the MA model of ageing. Yet, on the other hand, this culturing protocol should also favour mutations with antagonistic pleiotropic effects accumulating, which benefit early reproduction at the expense of maintenance, both in respect to the possibility of balancing selection maintaining AP mutations and due to AP mutations sweeping through the population (see Discussion above).

Conclusion

To our knowledge, this study is the first to ask whether the cytoplasmic genome may accumulate genetic variation that contributes to patterns of ageing according to an AP or MA model. As discussed above, our results are opposite to the patterns predicted under AP, but consistent with a modified MA model. Moreover, our results contribute to an increasing body of data that evokes a profound role for mitochondrial genes in ageing. Taken together, these findings thus suggest that MA may be a more important contributor to ageing than previously considered, when the effects of ageing are summed over both the nuclear and cytoplasmic genomes.

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