MOLECULAR ECOLOGY

Molecular Ecology (2013) **22**, 3539–3551 doi: 10.1111/mec.12353

# Complexity of the genetic basis of ageing in nature revealed by a clinal study of lifespan and *methuselah*, a gene for ageing, in *Drosophila* from eastern Australia

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## Abstract

**Clinal studies are a powerful tool for understanding the genetic basis of climatic adaptation. However, while clines in quantitative traits and genetic polymorphisms have been observed within and across continents, few studies have attempted to demonstrate direct links between them. The gene *methuselah* in *Drosophila* has been shown to have a major effect on stress response and longevity phenotypes based largely on laboratory studies of induced mutations in the *mth* gene. Clinal patterns in the most common *mth* haplotype and for lifespan (both increasing with latitude) have been observed in North American populations of *D. melanogaster*, implicating climatic selection. While these clinal patterns have led some to suggest that *mth* influences ageing in natural populations, limited evidence on the association between the two has so far been collected. Here, we describe a significant cline in the *mth* haplotype in eastern Australian *D. melanogaster* populations that parallel the cline in North America. We also describe a cline in *mth* gene expression. These findings further support the idea that *mth* is itself under selection. In contrast, we show that lifespan has a strong non-linear clinal pattern, increasing southwards from the tropics, but then decreasing again from mid-latitudes. Furthermore, in association studies, we find no evidence for a direct link between *mth* haplotype and lifespan. Thus, while our data support a role for *mth* variation being under natural selection, we found no link to naturally occurring variation in lifespan and ageing in Australian populations of *D. melanogaster*. Our results indicate that the *mth* locus likely has genetic background and environment-specific effects.**

*Keywords*: ageing, candidate gene, cline, Drosophila, lifespan, *methuselah*

*Received 27 July 2012; revision received 12 March 2013; accepted 16 March 2013*

## Introduction

Candidate genes for climatic adaptation are increasingly being identified from studies involving genome-wide scans, gene expression patterns, quantitative trait mapping, population and strain comparisons and comparative genomics (Hoffmann & Willi 2008; Hohenlohe *et al.* 2010; Turner *et al.* 2010; Kolaczkowski *et al.* 2011; Levine *et al.* 2011). While there are a large number of candidate genes now identified, in only a few cases have specific polymorphisms in these genes been linked to adaptive variation in traits. Examples in insects include *Cpo*, a gene that influences clinal variation in diapause (Schmidt *et al.* 2008), the *Dca* gene that influences clinal variation in body size (McKechnie *et al.* 2010; Lee *et al.* 2011b), the *InR* gene that influences a range of life history traits (Paaby *et al.* 2010), the *ebony* gene that affects clinal variation in pigmentation in *Drosophila melanogaster* (Telonis-Scott *et al.* 2011) and the *Pgi* polymorphism that influences dispersal and life history traits in butterflies (Hanski & Saccheri 2006; Saastamoinen *et al.* 2009; Wheat *et al.* 2011) as well as heat resistance in the montane willow beetle (Rank *et al.* 2007).

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Naturally occurring variation in the *methuselah* (*mth*) gene has been suggested to be under climatic selection and to contribute to genetic variation in lifespan, ageing and senescence (Schmidt *et al.* 2000). The *mth* gene encodes a G protein-coupled receptor (GPCR), and the Mth signalling pathway has a major effect on stress responses and longevity (Lin *et al.* 1998; Cvejic *et al.* 2004). In the first study on evolutionary dynamics at the *mth* locus and geographic patterns of allele frequency variation in natural populations of *D. melanogaster*, Schmidt *et al.* (2000) found strong evidence for non-neutral patterns of amino acid divergence in the intracellular and extracellular loop domains of the Mth protein. They also showed that the most common *mth* haplotype exhibited a strong latitudinal cline along the east coast of the United States, providing evidence for natural (climatic) selection operating at the *mth* locus. Duvernell *et al.* (2003) extended this study, providing further evidence that the clinal pattern in the most common *mth* haplotype is not the result of physical linkage to sites in the adjacent genes. These authors did not comprehensively examine clinal patterns in mortality and senescence along the same latitudinal gradient, nor did they directly test for an association between allelic variation at the *mth* locus and natural variation in lifespan; nevertheless Schmidt *et al.* (2000) suggested that *mth* affects ageing in natural populations. Paaby & Schmidt (2008) demonstrated functional variation in natural *mth* alleles using complementation tests, which lend support to allelic effects on lifespan. However, it remains unclear whether the wild *mth* allele that fails to complement the *mth-1* mutant allele is indeed the same allele that shows clinal variation in North America.

Geographic variation in lifespan has been described in *Drosophila* populations. Mitrovski & Hoffmann (2001) found that, under winter conditions in field cages, longevity showed a quadratic relationship with latitude in *D. melanogaster* from eastern Australia; lifespan decreased from the tropics (16 °S) to mid-latitudes, increasing again at the southernmost latitudes (41 °S). Schmidt & Paaby (2008) also found geographic variation in lifespan in *D. melanogaster* from eastern United States, but in contrast to Mitrovski & Hoffmann (2001) they found that lifespan increased with latitude, although this was examined over a shorter latitudinal gradient (25°–44°N) compared to the gradient considered by Mitrovski & Hoffmann (2001), and in the laboratory at 25 °C rather than in overwintering cages. Finally, in a study examining the effects of altitude on lifespan and senescence in *D. buzzatii*, Norry *et al.* (2006) found that lifespan decreased with altitude in females (but not males) when tested at 25 °C, whereas lifespan increased with altitude in males (but not females) when tested at 29 °C. In accordance with these patterns in lifespan, they also found that the rate of ageing increased in females at 25 °C and males at 29 °C. Tatar *et al.* (1997) also showed that lifespan decreased, and mortality rate increased, with increasing altitude in grasshoppers. While these studies suggest that lifespan, or a trait(s) genetically correlated with lifespan, is under selection in nature, they have not linked this variation with candidate genes for ageing.

Parallel evolution of clinal patterns in traits and gene frequencies in different continents provides evidence for adaptive evolution in response to climatic selection. Parallel *Drosophila* body size clines in Australia, South America, Africa and Europe indicate that climatic adaptation can produce the same phenotypic response at a continental scale (David & Bocquet 1975; Imasheva *et al.* 1994; Van 'T Land *et al.* 1999; Calboli *et al.* 2003; Arthur *et al.* 2008). Similarly, parallel clinal patterns particularly between Australia and North America suggest selection on molecular polymorphisms including the insertion/deletion variation in the insulin receptor gene (Paaby *et al.* 2010), several single nucleotide polymorphisms (Turner *et al.* 2010) and allozyme variation in alcohol dehydrogenase (Oakeshott *et al.* 1982) and esterase-6 (Oakeshott *et al.* 1981). However, inconsistent clinal patterns and genotype–phenotype associations across continents have also been noted. For instance, a strong latitudinal cline in starvation resistance in *D. melanogaster* populations from India (Karan *et al.* 1998) is absent in South America (Robinson *et al.* 2000) and in Australia (Hoffmann *et al.* 2001). More recently, Lee *et al.* (2011a) found that *cpo*, a candidate gene for climatic (clinal) adaptation that has been linked to diapause in *D. melanogaster* from North America (Schmidt *et al.* 2008), is not under climatic (clinal) selection and was not associated with diapause in populations of *D. melanogaster* from eastern Australia.

It remains unclear whether similar parallel clines occur in lifespan and in *mth* haplotype frequency in both North America and Australia, and whether *mth* is associated with naturally occurring variation in ageing outside of North American populations of *Drosophila*. To date, studies of naturally occurring genetic variation for lifespan and ageing have either focussed on candidate genes or on the phenotypes of lifespan and ageing. The study by Paaby & Schmidt (2008) is the only one so far to attempt to link functional variation in natural *mth* alleles to lifespan in nature. We have therefore investigated the link between clinal variation in the *mth* gene with genetically based variation in lifespan and ageing by examining clinal patterns in lifespan and mortality as well as *mth* haplotype frequencies in populations of *D. melanogaster* collected from along the east coast of Australia. We also examine levels of *mth* transcription along this same latitudinal gradient and test for a link

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between variation at the *mth* locus and lifespan by performing a single-generation selection experiment and association study.

## Materials and methods

### *Field collections of Drosophila melanogaster for clinal study of ageing and gene expression*

Populations of *D. melanogaster* were collected along the east coast of Australia in April-June 2008 from 17 locations along a transect extending from Cape Tribulation (16.26 °S) in far north Queensland to Sorrell (43.15 °S) in southern Tasmania (Table 1). Populations were collected as close to sea level as possible (at elevations < 100 m). Thirty isofemale lines (each line established with one field inseminated female) were generated for all populations. After two generations of laboratory culture, these isofemale lines were used to generate 17 mass bred populations, one from each collection site. The mass bred populations were maintained on an agar-dextrose-potato-yeast medium at 25 °C under 12:12 h light/dark cycle. Each population was maintained in five 250-mL bottles (approximately 200 flies per bottle) with nonoverlapping generations. The populations were maintained for 12 generations at a population size of at least 1000 flies per generation before being used in the clinal experiment described below.

**Table 1** Site locations for populations of *Drosophila melanogaster* used in this study

| Site | Latitude (°S) | Used in lifespan (L), expression (E) or genotype (G) analysis |
|---|---|---|
| Cape Tribulation | 16.26 | E |
| Atherton/Cairns | 17.20 | E |
| Innisfail | 17.52 | L, E, |
| Cardwell | 18.2 | L, E, G |
| Bowen | 19.97 | L, E |
| Finch Hatton | 21.13 | L, E, G |
| Rockhampton | 23.4 | L |
| Gladstone | 23.83 | G |
| Maryborough | 25.53 | L, E, G |
| Brisbane | 27.47 | G |
| Redland Bay | 27.6 | L |
| Ballina | 28.75 | L |
| Coffs Harbour | 30.37 | L, E, G |
| Port Macquarie | 31.42 | L, E, G |
| Hunter Valley | 32.77 | L |
| Gosford | 33.28 | L |
| Sydney | 33.87 | G |
| Crooked River Winery | 34.73 | L, E |
| Nowra | 34.87 | G |
| Bega | 36.67 | G |
| Pambula | 36.92 | L |
| Tilba Tilba Winery | 36.28 | E |
| Wandin | 37.77 | L, E |
| Metung | 37.88 | E |
| Dromana | 38.33 | G |
| North Tasmania | 41.23 | L, E, G |
| South Tasmania | 42.78 | G |
| South Tasmania | 43.15 | L, E |

Populations used for the lifespan and expression analyses were collected in 2008. Flies used in the genotype analysis were field-caught flies collected in 2005.

### *Experimental protocol for clinal longevity and mortality assay*

A generation before the clinal study, each mass bred population was subjected to a series of short egg-laying periods (between 6 and 18 h) in 40-mL vials containing 10 mL of medium to control for density effects. Approximately 50 male/female pairs eclosing from these short lay vials were placed into three separate empty 40-mL vials each containing a plastic spoon filled with a treacle-yeast-agar medium covered with a layer of live yeast paste to encourage oviposition. Experimental flies were obtained by leaving flies on spoons for approximately 12 h at 25 °C. Eggs were collected from these spoons and transferred at a density of 50 eggs per vial into 15 vials for each population. These vials were placed at 25 °C for development under 12:12 h light/dark cycle. Flies eclosing from these vials were collected as virgins and placed separately into holding vials for 3 days. Ten pairs of females and males were then placed into a vial (30 vials per population) and observed until all females had been observed to have mated once. Females and males were then separated and the males discarded. The once-mated females were used in the lifespan assay.

For each of the 17 clinal populations, 10 vials, each containing 30 once-mated females were set up and placed at 25 °C. The flies were transferred to fresh vials every day, and at each transfer, all vials were examined for dead flies. Flies were always kept in their replicate groupings throughout the experiment.

### *Coffs Harbour lines used in the methuselah association study*

For the *mth* association study, an independent population from the midpoint of the east coast transect (Coffs Harbour 30.22 °S) was collected in November 2009. Briefly, 60-field inseminated females were returned to the laboratory and used to set up isofemale lines. A mass bred population was established by pooling 10 virgin females and 10 males from each isofemale line. The mass bred population was allowed to freely recombine at a population size of at least 1000 flies at 25 °C

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12:12 h light/dark until the experiment was initiated at the $F_6$ generation of laboratory culture.

A generation before the association study, flies from the Coffs Harbour population were subjected to a series of short egg-laying periods (between 6 and 18 h) in 40-mL vials containing 10 mL of medium to control for density effects. Approximately 30 male/female pairs eclosing from these short lay vials were placed into 40-mL vials containing 10 mL of medium to further control for density effects. The flies eclosing from this second round of control density lays were used in the association study.

Flies eclosing from these vials were collected as virgins and placed separately into holding vials for 3 days. Ten pairs of females and males were then placed into a vial and observed until all females had been observed to have mated once for a total of 5500 females. Females and males were then separated and the males discarded.

Once-mated females were then placed in groups of 30 in vials and placed at 25 °C 12:12 hr light/dark. The flies were transferred to fresh vials every day until the last 500 females were alive. These last 500 females were frozen for genotyping (described below). A random sample of 500 females was also frozen at the beginning of the longevity study. These females were randomly collected from all vials of once-mated females. This approach allowed us to directly compare *mth* haplotype frequencies of the oldest surviving flies with those of the younger control individuals, thus confirming (or otherwise) an association between *mth* and lifespan.

### *Methuselah haplotype genotyping*

We designed a PCR assay to determine the ATATC haplotype of *mth* based on information reported in Schmidt *et al.* (2000). The ATATC haplotype described in Schmidt *et al.* (2000) is defined by a combination of specific nucleotide at five sites: #531 (A), #1048 (T), #1183 (A), #1189 (T) and #1244 (C). In Fig. 2 of Schmidt *et al.* (2000), all 10 individuals carrying the ATATC haplotype have 'T' at site #1048 and 'A' at site #1183; all remaining 12 non-ATATC sequences lack such a SNP combination. Hence, sites #1048 and #1183 are diagnostic for the 'ATATC' haplotype. Based on this observation, we designed allele-specific primers to match the SNP states of the two informative sites, separated by 134 nucleotides: T/C at site #1048 (r5.48, 3L: 344835) and A/C at site #1183 (r5.48, 3L: 344700). The two forward and two reverse primers are: Fwd-T: 5′-CTT TGTCATGGCCGCATTT-3′, Fwd-C: 5′-CTTTGTCATGG CCGCATTC-3′; Rev-T: 5′-GGCCAGAACGGTAATTCCT-3′, Rev-G: 5′-GGCCAGAACGGTTATTCCG-3′. The underlined letter at the 3′ end of each primer indicates the allele-specific nucleotide.

PCR amplification was carried out using the Roche LightCycler® 480 system on the 384-well format. The 10-L PCR contained 1 μL of Chelex-extracted DNA template (approximately 0.5% of the whole fly DNA), 0.4 μM of one forward and one reverse primer, 1× reaction buffer, 0.16 mM of dNTP mix, 2 mM of $MgCl_2$, 0.25 μL of the LightCycler® 480 High Resolution Melting Master (Roche), 0.05 units of IMMOLASE™ DNA polymerase (Bioline) and DEPC-treated water (Invitrogen) to make up the remaining volume. We used the touch-down PCR strategy to amplify the *mth* alleles: 95 °C for 10 min, 30 cycles of 95 °C for 5 s, 65–50 °C (reduced 0.5 °C per cycle) for 10 s and 72 °C for 15 s, and 20 additional cycles with an annealing temperature set at 50 °C. One fluorescence acquisition was obtained after each 72 °C step.

The presence of a haplotype in a gDNA sample was inferred by the successful amplification of haplotype-specific primer combination (i.e. Fwd-T/Rev-T, Fwd-T/Rev-G, Fwd-C/Rev-T, Fwd-C/Rev-G). As the experiment was performed in a real-time PCR instrument, the crossing point (CP) values could be obtained to distinguish between successful versus unsuccessful amplifications. This was carried out by examining the CP values of the samples for a given primer combination. Individuals with low CP numbers (i.e. amplified early) were scored 'haplotype presence', whereas individuals with high Cp values (i.e. amplified late) or no amplification was scored 'haplotype absence'. For example, an individual homozygous for the ATATC haplotype was deduced by the successful PCR amplification using primers Fwd-T and Rev-T and failure of all other primer combinations (Fwd-T/Rev-G, Fwd-C/Rev-T, Fwd-C/Rev-G). To validate the PCR assay, we sequenced seven random individuals and the Celera reference strain (Fig. S1, Supporting information). Universal primers (Mth-seq-F: 5′-CGATTTGTGGCAGA TATCCATTAGC-3′ and Mth-seq-R: 5′-ACCGTAA AAGTAGAGCATGGCTGAC-3′) were used to amplify a 460-bp gDNA fragment, which encompasses the two haplotype-defining nucleotides at sites #1048 and #1183. The homozygosity/heterozygosity status at each site was consistent with the results from the PCR assay (Fig. S1, Supporting information).

We applied this assay to screen populations from the same latitudinal transect described above but collected in 2005 (Table 1). This collection comprised a total of 220 field-caught females (average 18.3 per site, range 11–23 per site) from 13 populations between Cardwell, Queensland (18.25 °S) and Sorell, Tasmania (42.78 °S). The same assay was employed in the association study comparing the control and the longevity-selected populations. DNA was extracted using the Chelex/Proteinase K method as described in Lee *et al.* (2011a).

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### *Clinal analysis of mth gene expression*

Flies for gene expression experiments came from the 2008 clinal collection previously described in Lee *et al.* (2011a). Flies from the each clinal population were subjected to a 4-h egg-laying period on coloured agar medium. To control for larval density, 50 eggs were transferred from these spoons into individual vials each containing 7 mL of standard fly media. Vials were kept at 25 °C under constant daylight. Fifty four-day-old females per population were collected and stored in RNAlater solution (Ambion) at −70 °C for RNA extraction.

Total RNA was isolated using TRIZOL® Reagent (Invitrogen) and contaminating genomic DNA was removed using RQ1 RNase-Free DNase (Promega). The DNase-treated total RNA was purified using RNeasy Mini kit (Qiagen), and first-strand cDNA was synthesized using the SuperScript® III First-Strand Synthesis SuperMix (Invitrogen). Oligo-dT was used to prime the reverse transcription step. First-strand cDNA was diluted 10-fold in water for real-time RT–PCR.

Real-time PCRs were set up using the LightCycler® 480 High Resolution Melting Master (Roche) following manufacturer's instructions. The data (CP values) were acquired on the LightCycler® 480 (Roche) with the Absolute Quantification Module in the software package. The standard 'delta-delta CP' method was used to estimate relative expression (see Pfaffl 2001). The PCR amplification efficiency for a given primer pair was not empirically determined and was assumed to be identical across samples. The RT–PCR primers were as follows: mth-rt-F (5′-AAGCAGAAGCTGAACTCCGACA), mth-rt-R (5′- CC CAAGTTTGGTTGGATTGCG). Real-time PCR results were normalized using reference genes *RpL11* (*CG7726*) and *Gapdh2* (*CG8893*). The primers were RpL11-F (5′-CGATCTGGGCATCAAGTACGAT-3′) and RpL11-R (5′-TTGCGCTTCCTGTGGTTCAC-3′) for *RpL11*, Gapdh2-F (5′-GGTGCCGAATACATCGTGGAG-3′) and Gapdh2-R (5′-GGATGGGGCCGAGATGATAAC-3′) for *Gapdh2*. Cycling conditions were 95 °C for 10 min followed by 50 cycles of 95 °C for 10 s, 58 °C for 15 s and 72 °C for 15 s. Acquisition of data was carried out at each cycle immediately after the extension phrase. Five technical replications were performed per cDNA sample (clinal population) per gene.

### *Environmental variables*

Data for 19 climatic variables were obtained from WORLDCLIM (version 1.3, http://www.worldclim.org) (Table S1, Supporting information). We focussed on both temperature means and extremes, as well as measures of climatic variability, as recent work suggests that climatic variability may be as, if not more, important in determining responses to climatic change than changes in the mean of climatic variables. These variables are derived from the monthly temperature and rainfall values (obtained by interpolation of climate station records) in order to generate more biologically meaningful variables. We used the program DIVA-GIS (version 7.2.3.1, http://diva-gis.org/) to extract bioclimatic variables (spatial resolution of 2.5 arc min) for the collection sites for *D. melanogaster*.

### *Statistical analyses*

*Longevity and mortality.* To test for differences in mean longevity among the populations tested, a one-way analysis of variance (ANOVA) was performed, with population as a random effect. To test for any latitudinal patterns on mean longevity, regression analysis of mean longevity on latitude was performed. The data met the requirements of parametric analyses.

We used WinModest 1.0.2 (Pletcher 1999) to test for population differences and clinal patterns in mortality and senescence. First, we evaluated which of four different models (Gompertz, Gompertz–Makeham, logistic and logistic-Makeham) best described the temporal pattern of mortality in each vial, using log-likelihood tests. The Gompertz model describes an empirically observed pattern that mortality increases exponentially with age. Logistic models account for frequently observed deceleration of the rate of mortality late in life; however, when such deceleration equals zero, logistic models are reduced to Gompertz models (Pletcher 1999). Finally, Makeham models add a constant to account for age-independent mortality. In all cases in this study, the mortality function was best described by the Gompertz model $u_x = \alpha e^{\beta x}$, where $u_x$ is the predicted instantaneous mortality rate at age $x$, $\alpha$ is the Gompertz intercept, or frailty, and $\beta$ is the rate of senescence of the population (Finch 1990; Williams & Day 2003). This suggests that the fit of our data was not improved by adding the parameters that account for the effects of increased mortality early in life (Makeham models) or decelerated rate of senescence late in life (logistic models). We then performed sensitivity analyses to test for robustness of estimated model parameters for each vial using WinModest (Pletcher 1999; Maklakov *et al.* 2006). The use of maximum-likelihood estimations procedures as implemented in WinModest allows more accurate estimation of mortality parameters, especially with relatively low sample sizes (Promislow *et al.* 1996; Pletcher 1999; Pletcher *et al.* 1999). The Gompertz parameter for frailty and rate of senescence were estimated separately for each vial, and variance in frailty and rate of senescence among the populations examined was again

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analysed using one-way ANOVA with population as a fixed effect. To test for an effect of latitude on rate of senescence, we regressed $\alpha$ and $\beta$ on latitude.

*Clinal methuselah gene expression and haplotype patterns.* In order to test for any latitudinal patterns on the *methuselah* gene expression, regression analysis of expression on latitude was performed on population means. The same analysis was used for the frequency of the *mth* ATATC haplotype. We also explored whether environmental variables were significantly associated with the any clinal patterns in *mth* haplotype frequency and *mth* gene expression as well as mean longevity and lifespan parameters, along the latitudinal gradient. Principal component analysis (PCA) was run to reduce the 19 correlated temperature variables based on an analysis of the correlation matrix. Multiple linear regressions, using forward selection, were performed to examine the association between each of the PCs and traits/haplotype frequency.

*Methuselah–longevity association study.* A G-test was performed to compare the frequency of the *methuselah* haplotypes in the control (random) sample of flies and those selected for increased longevity. We tested for the power of this test by running simulations where longevity was assumed to have an underlying log normal distribution and then compared the amount of phenotypic variance that could be accounted for when 500 individuals were sampled randomly or at one extreme.

Tests for Hardy–Weinberg Equilibrium were performed for both the clinal and methuselah–longevity association studies. This was carried out by combining the three non-ATATC alleles into one group (i.e. non-ATATC). We therefore have three genotypes (ATATC homozygous, heterozygous and non-ATATC homozygous) for the HW tests.

For the 2005 clinal study, per population sample sizes were too small for accurate tests of HW (some genotypes < 5 individuals). Hence, we performed the test on the entire Australian collection ($N$ = 234). For the association analysis, we performed tests on the whole population ($N$ = 354) and separately for Control-only ($N$ = 167) and Selected-only ($N$ = 187) samples.

## Results

### Longevity and mortality

We found significant differences in mean longevity between the populations examined in this study ($F_{16,138}$ = 17.364, $P$ < 0.0001). Regression analysis for mean longevity revealed significant linear, quadratic and cubic components to the association with latitude (Table 2,

**Table 2** Associations between latitude and the traits examined

| | Linear component | | Quadratic component | | Cubic component | | Overall model | | |
|---|---|---|---|---|---|---|---|---|---|
| Trait | b ± SE | $P$ | b ± SE | $P$ | b ± SE | $P$ | $F$ ratio (d.f.) | $P$ | Adj. $R^2$ |
| Mean longevity | 16.24 ± 5.028 | 0.0066 | −0.5167 ± 0.018 | 0.0101 | 0.0051 ± 0.001 | 0.0178 | 13.54 (3,13) | 0.0003 | 0.702 |
| *B* parameter | −0.0091 0.003 | 0.0112 | 0.00012 4.79 × $10^{-5}$ | 0.011 | | | 4.65 (2,14) | 0.0343 | 0.459 |
| *mth* expression (adults) | 0.3619 × $10^{-6}$ ± 0.143 × $10^{-6}$ | 0.0256 | | | | | 6.354 (1, 13) | 0.0256 | 0.277 |
| *mth* haplotype | 0.0117 ± 0.0048 | 0.0350 | −0.0017 ± 0.0006 | 0.0212 | | | 4.831 (2,10) | 0.034 | 0.390 |

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Fig. 1). If we restrict our analysis to include only those populations that fall within the latitudinal range of 25–44 °S examined by Schmidt & Paaby (2008), we find a significant negative linear association with latitude – mean longevity decreases with increasing latitude (adj. $R^2 = 0.528$, $t = -3.648$, $P = 0.0045$, Fig. 1).

The mortality and senescence analyses revealed a significant effect of population on the Gompertz intercept ln(α) (frailty) ($F_{16,153} = 2.583$, $P = 0.0014$) as well as on the Gompertz rate of senescence β ($F_{16,153} = 1.907$, $P = 0.0235$). Because ln(α) and β tend to be negatively phenotypically correlated (Hughes 1995), we performed a separate analysis for the rate of senescence while taking into account the Gompertz intercept ln(α) as a fixed covariate. While ln(α) was indeed negatively correlated with β ($F_{1,16} = 1989.022$, $P < 0.0001$), the effect of population on the rate of senescence β remained significant ($F_{16,138} = 8.115$, $P < 0.0001$). Regression analysis revealed significant linear and quadratic components to the association between the rate of senescence β and latitude when all latitudes examined were considered (Fig. 2, Table 2).When we restrict our analysis to include those populations that fall within the latitudinal range of 25–44 °S examined by Schmidt & Paaby (2008), the association disappeared. The significant effect of latitude on age-specific mortality indicated by CPHR analysis can at least in part be explained by the significant differences in the Gompertz rate of senescence β. There was no significant association between the Gompertz intercept ln(α) (frailty) and latitude (adj, $R^2 = 0$, $t = 0.170$, $P = 0.867$).

## *Clinal methuselah gene expression study*

Using *RpL11* as the reference gene, regression analyses revealed a significant, positive linear association

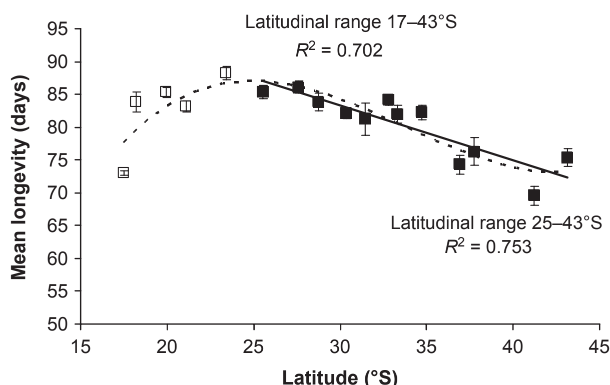


**Fig. 1** Regression of mean longevity (days) versus latitude. A quadratic regression is fitted to points from all latitudes, and a linear regression is fitted to points from latitudes 25 to 43 °S. Open squares represent tropical populations that were not considered by Paaby & Schmidt (2008).

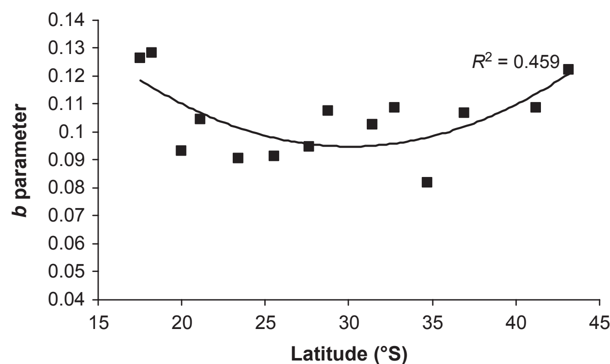


**Fig. 2** Regression of the Gompertz parameter for rate of senescence versus latitude. A quadratic regression is fitted to points from all latitudes.

between *mth* gene expression and latitude in adult females (Table 2, Figs 3 and S2, Supporting information) (adj. $R^2 = 0.277$; $t = 2.521$, d.f. = 1; $P = 0.026$). The clinal pattern in adults remained evident when *mth* expression was normalized to two reference genes (i.e. average of *RpL11* and *Gapdh2*) (adj. $R^2 = 0.212$; $t = 2.184$; d.f. = 1; $P = 0.048$), but was nonsignificant when only *Gapdh2* was used (adj. $R^2 = 0.098$; $t = 1.588$; d.f. = 1; $P = 0.136$; Fig. S2, Supporting information). No significant clinal signal was detected in either reference gene, based on reciprocal comparisons (i.e. *RpL11* relative to *Gapdh2* and *Gapdh2* relative to *RpL11*).

## *Clinal methuselah haplotype study*

Regression analysis revealed a significant positive association between haplotype frequency and latitude only when the mainland populations were considered (adj. $R^2 = 0.817$, $t = 6.759$, $P < 0.0001$; Fig. 4). When the Tasmanian populations were included, significant linear and quadratic components were evident; haplotype frequency decreased sharply in the Tasmanian populations. If we restrict our analysis to those populations that fall within the latitudinal range of 25–44 °S following

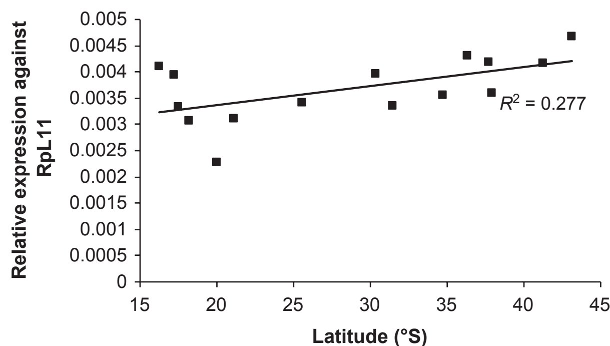


**Fig. 3** Regression of *mth* transcript in female adults versus latitude. Linear regression is fitted to points from all latitudes.

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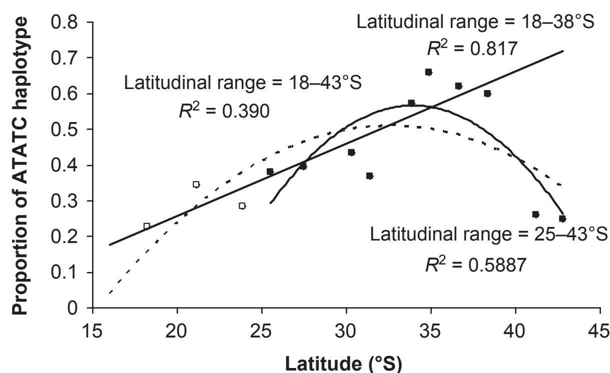


**Fig. 4** Regression of the *mth* haplotype versus latitude for populations collected in 2005. Quadratic regressions are fitted to points from all latitudes, and just those points representing latitudes examined by Paaby & Schmidt (2008). A linear regression is fitted to all points from latitudes 18 to 38 °S excluding Tasmania. Open squares represent tropical populations that were not considered by Paaby & Schmidt (2008).

Schmidt & Paaby (2008), the quadratic association with latitude increases in strength (Fig. 4).

There was no significant departure from Hardy–Weinberg Equilibrium ($\chi_1^2$ = 1.215, $P$ = 0.270).

### Clinal variation in traits and climate PCs

The PCA revealed four PCs that accounted for over 96% of variance in the climatic variables ($PC_1$ = 52.729%, $PC_2$ = 26.832%, $PC_3$ = 10.018% and $PC_4$ = 7.300%, Table S3, Supporting information). Annual, maximum and minimum temperature, as well as rainfall, contributed positively to $PC_1$, while climatic variability factors including diurnal range, annual temperature range and seasonality contributed negatively to $PC_1$ (Table S3, Supporting information). Diurnal and annual temperature range loaded positively onto $PC_2$, with negative contributions from precipitation, while temperature seasonality and temperature annual range made the largest contributions to $PC_3$. Diurnal range, isothermality and mean temperature of the wettest and driest months contributed to $PC_4$. (Table S3, Supporting information). Multiple regressions were performed between the PCs and mean longevity, *mth* gene expression and *mth* haplotype frequencies as these were the traits to show associations with latitude. Mean longevity was significantly associated with $PC_2$ and $PC_4$, and adult female *mth* expression was associated with $PC_1$ (Table 3). *Mth* expression in third instar larvae was not associated with any of the climatic variables (data not shown). Frequency of the *mth* haplotype was significantly associated with $PC_3$ and $PC_4$ when all populations including Tasmania were included; however, when only the mainland populations were considered, *mth* haplotype frequency was associated with $PC_1$ and $PC_2$. Both temperature and climatic variability thus seem to play a role in the observed clinal patterns, but different aspects of these climatic variables contribute to the traits examined.

### Methuselah-longevity association study

We found no association between longevity and *mth* haplotype when we performed a single-generation selection experiment and association study. None of the *mth* haplotypes differed in frequency between the control and selected, long-lived individuals ($G$ = 3.47, d.f. = 3, $P$ = 0.325; Fig. 5). Simulations suggest that we could have detected a difference between alleles accounting for 2 and 3% of the phenotypic variance.

There was no significant departure from Hardy–Weinberg Equilibrium when tests were performed on the whole population ($\chi_1^2$ = 1.147, $P$ = 0.284) or separately for Control-only ($\chi_1^2$ = 1.47, $P$ = 0.226) and Selected-only ($\chi_1^2$ = 0.136, $P$ = 0.712) samples.

## Discussion

Lifespan and age-specific mortality are key life history traits that vary significantly among natural populations (Promislow *et al.* 1996). The genetic architecture underlying lifespan has been shown to be highly complex and influenced by pleiotropy, epistasis and environment-specific effects (e.g. Pletcher *et al.* 2002; Spencer

**Table 3** Multiple regressions of traits against climate PCs: partial regression coefficients ± standard errors. Overall ANOVA, adjusted $R^2$ and F ratios are also shown

| | Mean longevity | *mth* expression (adults) | *mth* haplotype with Tasmania | *mth* haplotype without Tasmania |
|---|---|---|---|---|
| PC1 | | −0.000371 ± 0.00014* | | −0.139 ± 0.012** |
| PC2 | 2.687 ± 1.004* | | | −0.059 ± 0.011* |
| PC3 | | | 0.070 ± 0.025* | |
| PC4 | −3.299 ± 1.078* | | −0.118 ± 0.026** | |
| Overall ANOVA | 0.504, $F_{2,12}$ = 8.117* | 0.384, $F_{1,8}$ = 6.602* | 0.791, $F_{2,8}$ = 19.951** | 0.949, $F_{2,5}$ = 66.714** |

*$P$ < 0.05; **$P$ < 0.001.

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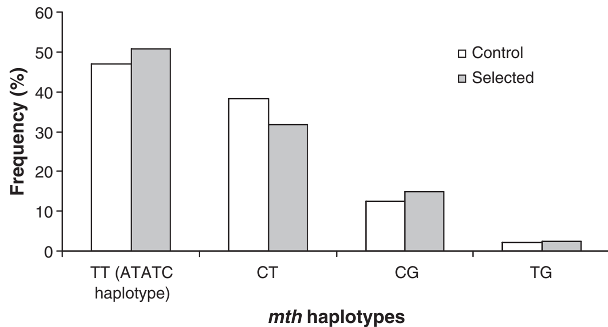


**Fig. 5** Overall proportion of *mth* haplotypes between the control and the longevity-selected populations. 'TT' denotes the 'ATATC' haplotype described in Schmidt *et al.* (2000).

*et al.* 2003; Baldal *et al.* 2006; Lai *et al.* 2007). Many genes that influence lifespan have been identified (e.g. Lin *et al.* 1998; Rogina *et al.* 2000; Clancy *et al.* 2001; Tatar *et al.* 2001; Hwangbo *et al.* 2004; Magwire *et al.* 2010) and some have become the focus of detailed studies on ageing and life history evolution (Schmidt *et al.* 2000; Paaby & Schmidt 2008; Paaby *et al.* 2010). However, few studies have explicitly linked naturally occurring variation in these candidate genes to naturally occurring genetic variation for longevity (but see De Luca *et al.* 2003; Carbone *et al.* 2006; Paaby & Schmidt 2008). In addition, studies demonstrating that variations in these candidate genes themselves are under selection in nature are limited. Recent work has implicated the gene *methuselah* (*mth*) as being under natural selection (Schmidt *et al.* 2000; Duvernell *et al.* 2003) and linked to variation in lifespan (Schmidt & Paaby 2008). These studies were based on populations of *D. melanogaster* from North America. Our analyses of samples from the Australian east coast suggest that: (i) clinal patterns in longevity and the *mth* haplotype frequency are different from the North American study, and (ii) that there is no evidence for a direct substantial association between the *mth* haplotype and lifespan on this particular genetic background and under the environmental conditions tested.

The motivation of this current study was to link naturally occurring variation in the *mth* gene with genetically based variation in lifespan and ageing. We did this by first testing for clinal variation in lifespan and ageing in *D. melanogaster* populations from eastern Australia. We showed that mean longevity of female *D. melanogaster* displayed a significant association with latitude, but that this association included nonlinear components. Specifically, we found significant linear, quadratic and cubic components to the association between mean lifespan and latitude. Lifespan increased from the tropics, levelling off at mid-latitudes, decreased towards the most southern temperate latitudes, increasing again at the southernmost sites. Nonlinear components to the association between mean lifespan, mortality and latitude have previously been reported for populations of *D. melanogaster* from the same latitudinal gradient in eastern Australia (Mitrovski & Hoffmann 2001; Hoffmann *et al.* 2003). However, the cline reported by Mitrovski & Hoffmann (2001) is quadratic (concave), which is almost opposite in pattern to that described in this study. Their study was performed in field cages over the colder winter months in southeastern Australia. Interestingly, clinal patterns in quantitative traits have been shown to change seasonally in Drosophila from eastern Australia. Specifically, Magiafoglou *et al.* (2002) found that clines in development time changed from concave prewinter to convex postwinter, while clines in viability changed from positive to negative and cold tolerance only showed significant clinal variation postwinter. It is therefore possible that the discrepancies between this current study and those of Mitrovski & Hoffmann (2001) reflect the role of seasonally changing selection pressures on lifespan and ageing in nature, or else reflect the substantial genotype–environment interactions associated with lifespan measurements.

The clinal pattern reported in this current study appears different to that described by Schmidt & Paaby (2008) for *D. melanogaster* from North America, where lifespan increased linearly with latitude based on three populations from a more restricted latitudinal range (25–44 °C) than the current study. When we restricted our analysis to a similar latitudinal range, we found a significant negative linear association with latitude, opposite in sign to the positive latitudinal pattern described by Schmidt & Paaby (2008). Norry *et al.* (2006) found that mean longevity decreased with altitude in *D. buzzatii* from Argentina, as did Tatar *et al.* (1997) in *Melanoplus* grasshoppers from California, suggesting that longevity may decrease with temperature as observed in the southern part of the latitudinal gradient considered here.

We also showed that the rate of senescence changed with latitude. Thus, the changes in mean lifespan observed in this current study can at least in part be explained by differences in the rate of mortality between the populations examined. Norry *et al.* (2006) and Tatar *et al.* (1997) also found population differences in rates of mortality that explained at least in part the observed altitudinal patterns in mean lifespan. It has been suggested (Lencioni 2004) that high-altitude environments should be associated with more hazardprone environments due to a combination of severity, seasonality, unpredictability and variability, and that this might explain the significant associations between altitude and mortality rate (Tatar *et al.* 1997; Norry *et al.* 2006). Whether this also applies to latitudinal clines is

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not known, although climatic variables examined in this study that includes variability and minimum temperature do show clinal patterns of variation, and these variables were associated with clinal variation in mean longevity and the *mth* haplotype frequency. Furthermore, mortality increases at lower temperatures in adult *D. melanogaster* when held in field cages over winter (Mitrovski & Hoffmann 2001). More hazardprone environments are predicted to affect lifespan by increasing intrinsic mortality and early fecundity at nonstressful temperatures (Finch 1990; Rose 1991; Partridge & Mangel 1999; Kirkwood & Austad 2000). Thus, if higher latitudes are associated with increased environmental harshness, both reduced lifespan and increased early fecundity in high-latitude populations might be expected. We have not examined fecundity in this current study, although fecundity varies positively with latitude in eastern Australia (Hoffmann *et al.* 2003). Of course, any inferences about selection based on observed clinal patterns and environmental associations need to be further investigated by directly measuring the strength and direction of selection operating on traits.

We then asked whether these patterns in mean longevity and mortality might be explained by clinal variation in *mth* transcript levels. We found a weak but significant clinal pattern in *mth* gene expression in 4-day-old females (Figs 3 and S2, Supporting information); transcript levels increased with latitude in adult females. Although clinal patterns in *mth* gene expression have not previously been examined, it has been suggested that down-regulation of *mth* is associated with extended lifespan. In particular, Pletcher *et al.* (2002) found significant down-regulation of *mth* when caloric restriction resulted in an approximately twofold increase in lifespan in *Drosophila*. These expectations regarding *mth* transcript levels are only partly met in the current study in the linear portions of the lifespan cline. The nonlinear clinal patterns cannot be explained by variation in *mth* gene expression, suggesting that, on the whole, *mth* may not be associated with differences in lifespan in the populations examined. Admittedly, the geographical pattern observed in *mth* expression is dependent on the choice of reference genes. A different pattern might emerge if other reference genes are used, or different life stages are being investigated.

When we examined the frequency of the *mth* haplotype that has previously been shown to display clinal variation in North America, we also found a significant association between the *mth* haplotype and latitude. However, this association included nonlinear components. Specifically, when all populations were considered, or when we restricted our analysis to those latitudes considered by Schmidt *et al.* (2000), we found strong linear and quadratic components to the clinal patterns (Fig. 4). When only the mainland Australian populations were included, the cline became significantly linear and positive, which did concur with the patterns reported by Schmidt *et al.* (2000). The reason for the sudden decrease in frequency in the Tasmanian populations in this current study is not known. While it has been suggested (Agis & Schlotterer 2001) that there is significant isolation and thus divergence between the Tasmanian and mainland Australia populations of *D. melanogaster*, subsequent studies suggest that this may not be the case for all markers (Gockel *et al.* 2002; Kennington *et al.* 2003).

The presence of significant, albeit complex, clinal patterns in lifespan and mortality, as well as *mth* transcript levels and haplotype frequency as reported in this study, suggests that they are the result of natural selection. However, the observed clines in lifespan and mortality did not seem to be explained by the observed clines in *mth* transcript or haplotype frequency. We then performed an association study which failed to link lifespan and *mth* haplotype, suggesting that the observed clinal patterns are the result of independent selection operating on lifespan and the *mth* locus although we did not have the power to detect small effects of this locus on lifespan. Thus, in the populations of *D. melanogaster* examined here and under the environmental conditions used, *mth* does not seem to represent an important gene for ageing, unlike in North American populations of *D. melanogaster* (Schmidt *et al.* 2000; Duvernell *et al.* 2003; Schmidt & Paaby 2008). Similarly, discordant results were observed by Lee *et al.* (2011a); they found that *cpo*, a candidate gene for climatic (clinal) adaptation that has been linked to diapause in *D. melanogaster* from North America, is under climatic (clinal) selection, but is not associated with diapause in populations of *D. melanogaster* from eastern Australia.

The absence of an association between naturally occurring variation at the *mth* locus and lifespan and senescence as noted in this study might be due to several factors. Specifically, genotype–environment interactions, genetic background and sex-specific effects have all been shown to significantly affect the genetic basis to longevity (Leips & Mackay 2000). Of particular relevance to this study is the fact that the $mth^1$ allele that was generated by P-element insertion and that was first linked to increased lifespan (Lin *et al.* 1998) itself has environment-specific effects under a range of laboratory conditions (Baldal *et al.* 2006). Perhaps associations between *mth* and lifespan depend on environmental conditions.

Inconsistencies in clinal patterns in complex traits across continents have previously been found (Long & Singh, 1995; Karan *et al.*, 1998; Robinson *et al.* 2000;

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Hoffmann *et al*. 2001; Lee *et al*. 2011a,b), and local adaptation might play a role in shaping clinal patterns of similar traits in different continents. For instance, high-latitude populations in North America are exposed to more extreme winter conditions than those from south-eastern Australia.

In conclusion, the results from this current study suggest that lifespan and *methuselah* are targets of natural selection. However, the absence of a significant association between the two indicates that *methuselah* is not a major factor influencing ageing in natural Australian *D. melanogaster* populations. Different patterns might emerge if other environmental conditions and populations were considered. These results and those of others highlight that even though populations of the same species might be confronted by similar climatic challenges, the phenotypic and genetic patterns to emerge may not always generalize across continents.

## Acknowledgements

CMS, BvH and AAH were supported by funding provided by the Australian Research Council through their Fellowship and Discovery Project Schemes. CMS and AAH were also supported by funding provided by the Science and Industry Endowment Fund. Monash University provided additional support to CMS.

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C.M.S. and A.A.H. devised the study. C.M.S., B.V.H., V.K., C.W.W. and S.F.L. designed and performed the experiments and collected the data. C.M.S., S.F.L. and C.W.W. analysed the data. All authors made contributions to the writing of the manuscript.

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## Data accessibility

Lifespan, haplotype and gene expression data: Dryad doi: 10.5061/dryad.11j35

Climate data: Supporting information

## Supporting information

Additional supporting information may be found in the online version of this article.

**Fig. S1** PCR assay validation.

**Fig. S2** Regression of relative mth transcript level against latitude.

**Table S1** Climatic data from along the east coast of Australia.

**Table S2** Principal Components analysis on temperature and variability data.

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