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Thermal plasticity in *Drosophila melanogaster* populations from eastern Australia: quantitative traits to transcripts

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thermal stress.

Abstract

The flexibility afforded to genotypes in different environments by phenotypic plasticity is of interest to biologists studying thermal adaptation because of the thermal lability of many traits. Differences in thermal performance and reaction norms can provide insight into the evolution of thermal adaptation to explore broader questions such as species distributions and persistence under climate change. One approach is to study the effects of temperature on fitness, morphological and more recently gene expression traits in populations from different climatic origins. The diverse climatic conditions experienced by *Drosophila melanogaster* along the eastern Australian temperate-tropical gradient are ideal given the high degree of continuous trait differentiation, but reaction norm variation has not been well studied in this system. Here, we reared a tropical and temperate population from the ends of the gradient over six developmental temperatures and examined reaction norm variation for five quantitative traits including thermal performance for fecundity, and reaction norms for thermotolerance, body size, viability and 23 transcript-level traits. Despite genetic variation for some quantitative traits, we found no differentiation between the populations for fecundity thermal optima and breadth, and the reaction norms for the other traits were largely parallel, supporting previous work suggesting that thermal evolution occurs by changes in trait means rather than by reaction norm shifts. We examined reaction norm variation in our expanded thermal regime for a gene set shown to previously exhibit GxE for expression plasticity in east Australian flies, as well as key heat-shock genes. Although there were differences in curvature between the populations suggesting a higher degree of thermal plasticity in expression patterns than for the quantitative traits, we found little evidence to support a role for genetic variation in maintaining expression plasticity.

Introduction

Phenotypic plasticity is the ability of a single genotype to generate diverse phenotypes in response to environmental variation (West-Eberhard, 2003). This flexibility is widespread and is predicted to be adaptive when the altered phenotype parallels the native optimum phenotype, but how plasticity impacts adaptive evolution is debatable because of limited empirical data (Ghalambor *et al.*, 2007). Temperature is a ubiquitous factor affecting organismal fitness and distributions, which are often limited to specific thermal ranges to maintain biochemical stability and metabolic activity (David & Tsacas, 1981; Cossins & Bowler, 1987; Hochachka & Somero, 2002). This is particularly true for ectotherms, as their thermal environment dictates the maintenance of homeostasis, body temperature, adult size and ultimately fitness (Huey, 1982; Stevenson, 1985; Angilletta & Dunham, 2003; Angilletta, 2009). The impact of enzyme thermodynamics on thermal sensitivity underpins opposing hypotheses on the evolution of optimal phenotypes in warm- and cold-adapted organisms where it is proposed that 'hotter is better', because

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higher temperatures expedite chemical reactions (Angilletta *et al.*, 2010). The interplay between temperature and plasticity is highly topical in thermal biology research, with a focus on understanding the mechanisms of plasticity and role in phenotypic evolution, population/species diversity and distributions, and persistence in a changing climate (reviewed in Sgrò *et al.*, 2016).

Thermal reaction norms are typically used to describe and compare the effects of temperature (Huey & Stevenson, 1979; Scheiner, 1993) and are a useful measure of the scale and direction of a plastic response. While reaction norms describe the effect of temperature on final trait values, a second measure of thermal plasticity, performance during thermal exposure, can be illustrated by the thermal performance curve (Kingsolver *et al.*, 2004; Angilletta, 2009). The properties defined by the performance curve permit biologically important inferences regarding the thermal optimum (T_{opt}), the temperature at which performance is maximal (P_{max}), and performance breadth and thermal limits (Angilletta *et al.*, 2002). These graphical and mathematical models can be applied empirically to study the evolution of thermal plasticity using different approaches (summarized in Fragata *et al.*, 2015). A common approach in ectotherms is to compare developmental acclimation across a thermal range in populations/species from different climatic origins (Trotta *et al.*, 2006; Yamahira *et al.*, 2007; Liefting *et al.*, 2009; Berger *et al.*, 2013; Klepsatel *et al.*, 2013a; Fallis *et al.*, 2014; Phillips *et al.*, 2014; Zhao *et al.*, 2015). In this framework, differences in the slopes and/or thermal breadth and optima of reaction norms may provide evidence for geographic (i.e. genetic) variation in the direction and/or the magnitude of plasticity (Kingsolver *et al.*, 2004). Similarly, nonadditive effects of the genotype in different environments known as genotype-by-environment interactions (G × E) can indicate genetic variation for plasticity (Scheiner, 1993; Price *et al.*, 2003; DeWitt & Scheiner, 2004).

Drosophila melanogaster is ideal for studying thermal adaptation; this species is viable across a wide temperature range (reviewed in Hoffmann, 2010) and exhibits parallel clines in quantitative fitness and morphological traits, chromosome inversions, DNA polymorphisms, gene expression and other traits (David *et al.*, 1977; James *et al.*, 1997; Azevedo *et al.*, 1998; Land *et al.*, 1999; Gibert & Huey, 2001; Hoffmann & Weeks, 2007; Adrion *et al.*, 2015). Clinal patterns may arise where there are spatially continuous changes in traits, and taking population structure into account, can reflect natural selection to climatic conditions such as temperature (Endler, 1977; Hoffmann & Weeks, 2007). The eastern Australian temperate-tropical latitudinal gradient is an excellent resource to study intraspecific local adaptation given the diverse local climates (Hoffmann & Weeks, 2007), clines in thermal tolerance (Hoffmann *et al.*, 2002; Sgrò *et al.*, 2010) including a thermal candidate gene *Hsrω* (Cockerell *et al.*, 2014), gene expression (Lee *et al.*, 2011; Telonis-Scott *et al.*, 2011) and thermal phenotypic plasticity (Sgrò *et al.*, 2010; Telonis-Scott *et al.*, 2011). Rapid latitudinal shifts in DNA polymorphisms on the Australian east coast may also serve as indicators for climate change (Umina *et al.*, 2005).

Drosophila thermal plasticity research more generally has focused on quantitative phenotypes including morphometrical traits such as bristle number, body size, body colouration and ovariole number (Delpuech *et al.*, 1995; Morin *et al.*, 1999; Moreteau *et al.*, 2003; Gibert *et al.*, 2004; Klepsatel *et al.*, 2013a), and fitness traits such as thermal performance for fecundity (Klepsatel *et al.*, 2013a) and impacts of thermal regime on thermotolerance (Hoffmann & Watson, 1993; Overgaard *et al.*, 2011). Recent high-throughput –omics platforms assess tens of thousands of transcript-level phenotypes simultaneously, and there has been increasing interest in genome-wide thermal expression plasticity correlated with geographic origin and in the role of spatially varying selection in maintaining transcriptome-level variation between populations and species (Levine *et al.*, 2011; Zhao *et al.*, 2015). Genomewide reaction norms have also been used to identify genes with common regulatory architecture and functional roles (Chen *et al.*, 2015).

Thermal variation is also thought to impact performance; Levins (1968) proposed that widespread species experience greater thermal heterogeneity than restricted species, leading to predictions of broader performance breadth in temperate verses tropical *Drosophila* (Overgaard *et al.*, 2011). The limited empirical data, however, is inconclusive; several studies show that while *Drosophila* quantitative traits are highly plastic, differences in some fitness traits are driven by trait mean values rather than differences in reaction norms (plasticity) (Hoffmann & Watson, 1993; Delpuech *et al.*, 1995; Overgaard *et al.*, 2011; Cooper *et al.*, 2012; Klepsatel *et al.*, 2013a). Conversely for morphology, plasticity may be a factor underpinning differences between tropical and temperate *Drosophila* in traits such as size colouration (David *et al.*, 1997; Morin *et al.*, 1999). Molecular phenotypes are also highly plastic; developmental acclimation impacted over 80% of the expressed genes over a broad thermal range in inbred *D. melanogaster* adults (Chen *et al.*, 2015). However, comparative thermal plasticity expression data in outbred populations from different climatic origins is so far limited to two extreme rearing temperatures (Levine *et al.*, 2011; Zhao *et al.*, 2015). There is evidence, however, for G × E for a number of genes suggesting the maintenance of genetic variation for thermal plasticity related to latitude in *D. melanogaster* (Levine *et al.*, 2011; Zhao *et al.*, 2015), but to a lesser extent in *D. simulans* (Zhao *et al.*, 2015). However, the limited number of thermal environments used in these studies provides limited

insight into the relative contribution of plasticity vs. trait mean divergence in climatic adaptation (Sgrò *et al.*, 2016).

In this study, we utilize the well-established 'cline-end' sampling strategy (e.g. Hoffmann & Watson, 1993; Morin *et al.*, 1999; Trotta *et al.*, 2006; Levine *et al.*, 2011) to comprehensively survey thermal plasticity across a wide range of temperatures in a tropical and temperate population of *D. melanogaster* from eastern Australia. Reaction norm variation in quantitative and molecular traits across several thermal environments has not been well studied, and we address this by assessing a test set of 28 fitness, morphological and molecular traits in outbred populations from opposing ends of the same climatic gradient and ask whether mean performance and reaction norms differ according to climatic origin. For the fitness trait fecundity, we examined key parameters of thermal performance, thermal optima, maximum output and breadth. We examined stress resistance variation using standard measures of heat and cold tolerance and examined egg-to-adult viability and body size reaction norms. Utilizing our wider thermal range, we also examined a test set of genes identified from whole transcriptome studies that have previously shown evidence for geographic and/or G × E for thermal plasticity (Levine *et al.*, 2011; Chen *et al.*, 2015; Zhao *et al.*, 2015) to explore potential patterns of spatial selection maintaining genetic variation for molecular plasticity in a comparative framework.

Materials and methods

Drosophila melanogaster collection and maintenance

Drosophila melanogaster were collected using banana baits from Melbourne (temperate; 37.8136°S, 144.9631°E) and Innisfail (tropical; 17.5236°S, 146.0292°E), Australia, in March and May 2013, respectively. From each collection site, 30 wild females were set up in the laboratory as separate isofemale lines. At generation F_2 of laboratory culture, mass-bred populations were established by pooling 10 virgin males and females from each isofemale line (600 flies per population) into two 250-mL bottles containing potato–dextrose–agar medium. The populations were expanded and maintained in sizes of at least 1000 individuals at 25 °C under 12 : 12-h light:dark cycle for 7–21 generations before transfer to the six thermal regimes (Table S1).

Thermal regime experimental design

The experimental populations were initiated at 25 °C in bottles containing standard medium (described above) by placing approximately 250 flies per bottle and allowing females to oviposit for two hours prior to removal of all adults. The bottles were then placed into one of six environmental chambers (MLR-325H; Panasonic, Kadoma, Japan) set to 12 : 12-h light:dark at 16 °C, 18 °C, 22 °C, 25 °C, 28 °C and 30 °C. The developmental temperatures were chosen to represent the range of temperatures that *D. melanogaster* experience in their thermal range permissible to reproduction and development (David *et al.*, 1997). Three bottles per population were placed into each cabinet. Oviposition was staggered across several days to synchronize eclosion based on previous assessment of development rates at the different temperatures, thus permitting simultaneous assessment of all population/temperature combinations.

Quantitative trait phenotyping

Fecundity

Daily female fecundity was examined over a 10-day period. The flies were cultured as for the thermal tolerance assays (described below); however, imagoes were collected and sorted by sex while still virgin. Thirty pairs of female and male flies from each thermal regime from each population were then placed into individual vials with medium and mated for 24 h prior to the commencement of the experiment. Each day, the pairs were aspirated into a new vial containing a spoon with blue-dyed medium and 10-μL activated yeast paste (1 : 3 live yeast:water). The number of eggs each female laid per 24-h period was recorded. Absolute fecundity was determined to be the mean cumulative number of eggs each female laid.

Heat knockdown assay

Heat knockdown time (Hoffmann *et al.*, 2002) was used to assess thermotolerance in 4- to 5-day-old females. Imagoes were collected into mixed-sex cohorts and mated for at least 48 h. At 3–4 days post-eclosion, females were separated into groups of 20 using aspiration without CO_2. The females were maintained in 10-dram vials with medium at their respective developmental temperatures prior to the heat assay. Immediately before the assay, the vials were moved to room temperature and individual females were aspirated into 5-mL glass vials and then immersed in a preheated water bath set to 38.5 °C. Heat knockdown was scored to the nearest second when the fly had become incapacitated. Approximately 30 flies from each population/temperature combination were scored across three replicate assays (blocks), each with approximately 10 flies per population/temperature.

Chill coma recovery

We assessed cold tolerance in 4- to 5-day-old females using a chill coma recovery assay (Gibert *et al.*, 2001). Flies were reared and prepared as for heat knockdown. For the assay, individual females were transferred into empty 1.7-mL Eppendorf tubes and immersed in a

prechilled 0 °C glycol bath and exposed for 6 h. Flies were then removed and allowed to recover at 25 °C, where the time taken (in seconds) for each fly to right itself (stand upright) was recorded. Flies that had not recovered at three hours post-stress were excluded from the analysis (four flies). Approximately 30 flies from each population/temperature combination were assessed simultaneously.

Egg-to-adult viability

Egg-to-adult viability at each of the six developmental temperatures was determined for each population. At 25 °C, approximately 1000 flies were placed onto Petri plates containing medium and *ad libitum* yeast paste and females were allowed to oviposit for two hours. Twenty eggs were then transferred into vials containing medium, and 15 vials were set up per population/temperature combination. As progeny eclosed, they were counted and collected into vials containing medium. At 4–5 days, the females were frozen and stored at −20 °C for the body size measurements.

Body size

Wing size was calculated as a proxy for body size (David *et al.*, 1997). The right wing from 600 females (50 per population/temperature) was removed using forceps, mounted onto a glass slide with double-sided tape and secured with a cover slip. Where the right wing could not be mounted, the left was used instead. Each wing was then photographed using a Leica M80 stereo microscope (Leica, Heerbrugg, Switzerland) with a digital camera attached. Eight wing vein landmark positions were obtained (Fig. S1), and their *x*- and *y*-coordinates determined using tpsDIG software version 2.17 (Rohlf, 2006). Wing area was then measured as centroid size (the square root of the sum of the squared distances from each landmark to the centroid) and calculated using CoordGen8 software (Sheets, 2003).

Quantification of transcript abundance

Candidate gene rationale

To examine the impact of thermal regime and population of origin on molecular phenotypes from the extremes of the same latitudinal gradient, we chose 18 genes according to the following criteria: (i) involvement in thermal tolerance [*Hsf, Hsrω* and *Hsp70Aa* (Hoffmann *et al.*, 2003)] (ii) evidence of population-specific expression variation [*Cyp6g1*, *CG9509* and *CG7214* (Hutter *et al.*, 2008)] and evidence of G × E for expression [temperature-by-populations interactions; *Cyp6a17*, *Cyp6a23*, *Lectin-galC1*, *lectin-33A*, *mag*, *Mal-B1*, *Mur29B*, *CG6912*, *CG10910*, *CG30083*, *CG33346* and *CG42807* (Levine *et al.*, 2011)]. Transcripts of interest were chosen based on published literature at the study outset; that is, Chen *et al.* (2015) and Zhao *et al.* (2015) had not been published but were incorporated into the cross-study comparison *post hoc*. We examined the multiple isoforms of *Hsf* and *Hsrω* in more detail given the evidence of isoform-specific thermal and/or population responsiveness (Fujikake *et al.*, 2005; Johnson *et al.*, 2011; Lakhotia, 2011; Cockerell *et al.*, 2014). We designed primers to target a common region of all *Hsf* transcripts as well as four isoform-specific primer sets to partition expression of *Hsf-RA*, *RB*, *RC* and *RD*. The *Hsrω* locus produces multiple nuclear and cytoplasmic long noncoding RNAs, and we examined the longer nuclear transcripts as an isoform subset separately to the shorter cytoplasmic subset. A total of 23 transcripts/transcript subsets were examined in 18 genes. Primer sequences were designed using PRIMER-BLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast/; Ye *et al.*, 2012; Table S2).

Fly collection, mRNA extraction, cDNA synthesis and real-time PCR

The flies for the gene expression assays were collected, sorted by sex and maintained as described for the thermotolerance assays. At day 4–5 post-eclosion, groups of 20 female flies were transferred into 1.7-mL Eppendorf tubes, immediately snap-frozen in liquid N_2 and stored at −80 °C. Five replicates from each population/temperature combination were collected (60 samples in total).

mRNA was isolated from pools of 20 females per sample using a Dynabeads® mRNA DIRECT™ Purification kit (Life Technologies, Carlsbad, CA, USA). Concentrations were determined using the NanoDrop spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA), and integrity was assessed using 2% agarose gel electrophoresis. cDNA was synthesized in a 20 μL volume from 50 ng mRNA using a Transcriptor High Fidelity cDNA Synthesis kit (Roche, Basal, Switzerland) according to the manufactures' instructions. Two kits were used from separate batches, and the samples reverse-transcribed from each kit were recorded and incorporated into the statistical analyses. The above steps were performed on small, randomized batches of samples. Real-time PCR was performed in 384-well plates using a *Roche* Lightcycler® 480 and SYBR Green chemistry in a 10 μL reaction. Transcripts were amplified using Lightcycler® 480 SYBR Green 1 master-mix where each well contained 5 μL PCR buffer, 4 μL 1 μM primer mix and 1 μL 1 : 9 diluted cDNA. Samples were quantified in duplicate (technical replicates), with five biological replicates analysed per population/temperature combination, except for *Cyp6g1* and *Lectin-galC1*-which were analysed using three biological replicates. Each biological replicate containing all population/temperature combinations was run together on a plate along with three 'housekeeping' genes, *RpL11, Gapdh2 and CycK*. Each housekeeper was verified for both population and thermostability prior to the gene expression assays using two-way analysis of variance (ANOVA; data

not shown). Transcript abundance was quantified relative to the geometric mean (GM) of the housekeepers using the formula: transcript of interest (TOI) = $2^{(GM-TOI)}$.

Reaction norms and performance curves analyses

Fecundity data were analysed as thermal performance curves by assessing BIC of Gaussian functions (Angilletta, 2006) fitted to fecundity (F) in the form:

$$F = a \exp\left[-0.5\frac{(T-b)^2}{c^2}\right]$$

where a is maximum fecundity (u_{max}), b is optimal temperature (T_{opt}), c is the standard deviation of the mean (performance breadth: $T_{br} = 2c$), and T denotes a given experimental temperature. Both thermal performance curves and reaction norm functions were fitted using nls() in R (v3.2.0).

For the heat knockdown, chill coma recovery, viability, body size and transcript abundance data population trait means were related to developmental temperature (i.e. average population-level reaction norms) by fitting first- to fourth-order polynomial functions. Functions with minimal Bayesian information criterion (BIC; Table S3) were selected as best-fitting models (Schwarz, 1978).

Statistical analysis

We next used ANOVA to examine the effects of population and temperature regime on the trait means. Model selection for each trait was determined using diagnostics including Shapiro–Wilk tests for residual normality and Levene's test of equal variances (SAS v9.4, SAS Institute Inc., Cary, NC, USA; PROC UNIVARIATE and GLM, respectively).

For the fecundity data, a mixed model was fit with REPEATED/SUBJECT = individual (population) and the GROUP = temperature statements to account for unequal variances driven by temperature differences (PROC MIXED, SAS V9.4). The fecundity data were also analysed using an analysis of covariance (ANCOVA) with population and temperature as fixed factors and wing size as a covariate. Following Klepsatel *et al.* (2013a,b), fecundity thermal performance curve parameters obtained by fitting Gaussian functions were bootstrapped to determine their standard error. To do this, fecundity data were first simulated based on parameter estimates obtained by fitting Gaussian functions. T_{opt}, u_{max} and T_{br} estimates were then calculated and the process repeated 1000 times for each population.

A fully factorial, two-way general linear-model ANOVA was fit to the heat knockdown and body size data with the fixed effects of population and temperature. A three-way fixed-effects general linear-model ANOVA was fit separately to each gene/transcript, with the fixed effects of cDNA synthesis kit (kit), population and temperature, and two-way interactions between the three main terms. For all transcripts, the effect of kit was stable across the populations and temperatures; therefore, the models were reduced to include only the interaction between temperature and population. Both the heat knockdown and gene expression data were log-transformed to improve normality. The chill coma recovery data were positively skewed and were analysed using a two-way generalized linear model with gamma distribution (link = log). The egg-to-adult viability data analysed using a generalized linear model (link = logit) to account for bimodal distributions with the fixed effects of population and temperature. For the quantitative traits across all temperatures, pair wise-planned contrasts were performed within each population (15 comparisons) and between-population comparisons were performed for the six temperatures (six comparisons) with correction for multiple tests using a false discovery rate (FDR) approach at FDR < 0.05 (Benjamini & Hochberg, 1995).

Finally, we additionally analysed thermal reaction norms for all quantitative and transcript phenotypes (barring fecundity) using either linear or nonlinear regression from the BIC best curve fitting models. For traits with linear reaction norms, linear regression was performed on each population separately with temperature as a continuous factor. For traits with quadratic, cubic or quartic reaction norms, nonlinear regression was performed on the populations separately. Each nonlinear regression had either two, three or four continuous factors (quadratic: temperature and temperature^2; cubic: temperature, temperature^2 and temperature^3; quartic: temperature, temperature^2, temperature^3 and temperature^4). The raw data were fit for each model, and the transcript data were fit with kit as a main factor. The least-squares means (LS means) derived from the full ANOVA models for all traits are shown for illustrative purposes.

Results

Effects of thermal regime on quantitative traits

Mean daily fecundity was significantly affected by temperature, population, and the interaction between them (Table 1). For each population separately, all pairwise temperature comparisons were performed (15 comparisons), but for interpretative ease, between-population comparisons were restricted to the same temperature (six comparisons). Within-population pairwise-planned contrasts were significant for all comparisons except 25 °C vs. 28 °C for both tropical and temperate females (FDR < 0.05, Fig. 1). On average, the tropical females were significantly more fecund at 16 °C, whereas the temperate females were more fecund at all rearing temperatures except 30 °C (FDR < 0.05, Fig. 1). We also

Table 1 Results for two-way ANOVAS on the on the fixed effects of developmental temperature, population (temperate and tropical) and the interaction term for fecundity*, heat knockdown time and body size. Significant terms are shown in bold.

Trait	Source of variation	d.f.	SS	*F*	*P* value
Fecundity	Temperature	5	–	3802.7	**1E-15**
	Population	1	–	7.52	**0.006**
	Temperature × Population	5	–	23.6	**1E-15**
	Error		–		
Heat	Temperature	5	39.832	72.06	**1E-15**
	Population	1	0.421	3.81	**0.052**
	Temperature × Population	5	0.812	1.47	0.199
	Error	344	38.028		
Body size	Temperature	5	14.081	1304.74	**1E-15**
	Population	1	0.432	199.81	**1E-15**
	Temperature × Population	5	0.013	1.25	0.287
	Error	588	1.27		

*Fecundity data were fit using a mixed-model ANOVA to account for unequal variances and uses a likelihood-based estimation where sum of squares (SS) are not output.

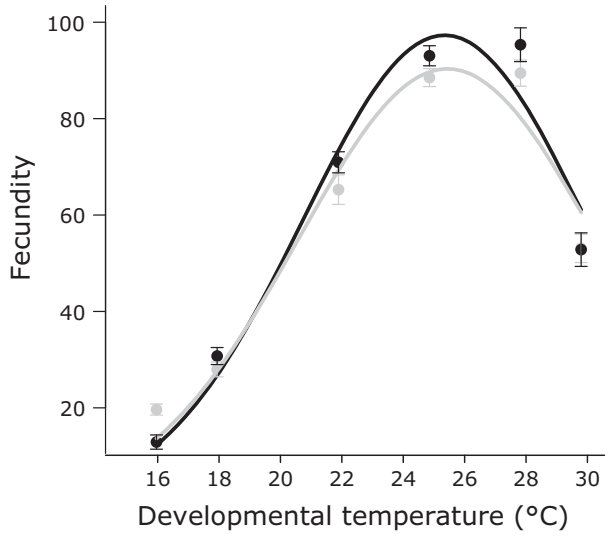

Fig. 1 Thermal performance curves for average daily fecundity in temperate (black) and tropical (grey) flies. Error bars represent standard error of the least-squares means.

examined the effect of body size on mean daily fecundity using ANCOVA and found no effect of body size, but significant effects of temperature, population and the interaction between them (temperatature: $F_{5,346} = 1252.5$, $P < 0.0001$; population: $F_{1,346} = 7.94$, $P < 0.01$; temperature-by-population: $F_{5,346} = 23.34$, $P < 0.0001$).

Fecundity performance curve parameter estimate analyses showed that the tropical and temperate females did not differ in their optimal temperatures (T_{opt} temperate: 25.5 °C ± 0.12 °C; tropical: 25.59 °C ± 0.14 °C) or performance breadth (T_{br} temperate: 9.33 °C ± 0.28 °C; tropical: 9.86 °C ± 0.32 °C). However, the populations did differ in their maximum fecundity with temperate females producing more eggs on average per day than tropical females (u_{max} temperate: 97.29 ± 2.08 eggs/day; tropical: 90.32 ± 1.87 eggs/day).

Rearing temperature significantly affected trait means for heat knockdown and body size (Table 1), chill coma recovery and egg-to-adult viability (Table 2). Population of origin also significantly impacted mean chill coma recovery time and body size (Tables 1 and 2) and marginally for mean heat knockdown time (Table 1, $P = 0.0518$). The impact of thermal regime on the trait means was similar between the populations, evidenced by the lack of temperature-by-population interactions (Tables 1 and 2) and qualitatively parallel reaction norms (Fig. 2a–d).

For heat knockdown, within-population pairwise-planned contrasts were significant for all comparisons except 16 °C vs. 18 °C, 22 °C vs. 25 °C and 28 °C vs. 30 °C, and the same result was observed in both populations (Fig. 2a. FDR < 0.05 for all other comparisons). Between-population contrasts were significant only at 16 °C and 18 °C due to higher knockdown resistance in temperate females compared to tropical females

Table 2 Results for two-way generalized linear-model ANOVAS on the fixed effects of developmental temperature, population (temperate and tropical) and the interaction term for chill coma recovery time and egg-to-adult viability. Significant terms are shown in bold.

Trait	Source of variation	d.f.	χ^2	*P* value
Cold	Temperature	5	281.3	**1E-15**
	Population	1	17.65	**2.7E-05**
	Temperature × Population	5	7.9	0.162
Viability	Temperature	5	31.88	**6.3E-06**
	Population	1	0.68	0.41
	Temperature × Population	5	7.05	0.217

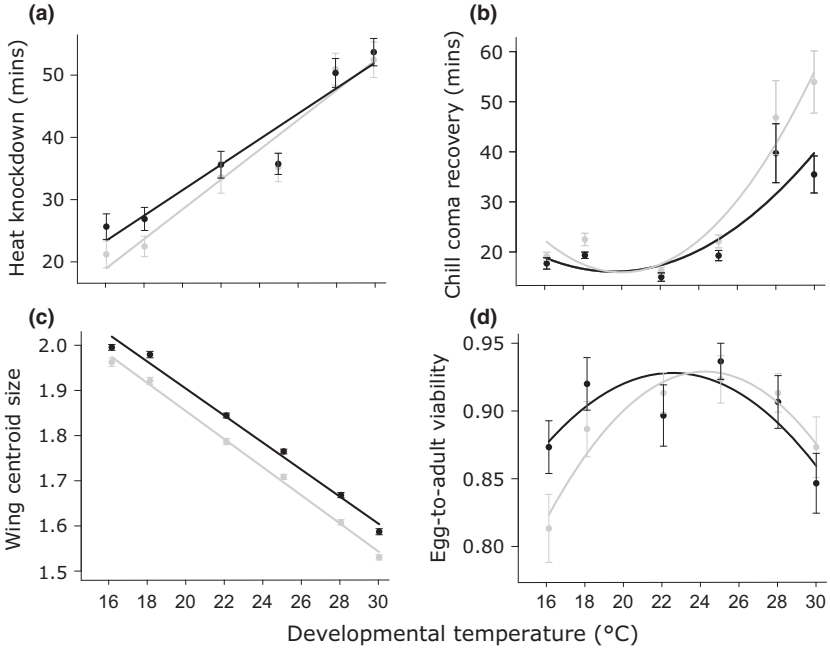

Fig. 2 Thermal reaction norms for quantitative trait population means in temperate females (black) and tropical (grey) females from eastern Australia. (a) Linear reaction norms for heat knockdown resistance, (b) quadratic reaction norms for chill coma recovery, (c) linear reactions norms for body size (approximated via wing centroid size) and (d) quadratic reaction norms for egg-to-adult viability. Error bars represent standard error of the least-squares means.

(Fig. 2a. FDR < 0.05 and < 0.1, respectively). For chill coma recovery, rearing temperature reduced recovery time at the high temperature extremes; within-population pairwise-planned contrasts were significant for contrasts except 16 °C vs. 18 °C, 16 °C vs. 22 °C, 16 °C vs. 25 °C, 18 °C vs. 25 °C and 28 °C vs. 30 °C (Fig. 2b. FDR < 0.05 for all other comparisons). The temperate females were more chill coma resistant than tropical females only at 30 °C (FDR < 0.05, Fig. 2b). Within populations, body size was significantly different between all temperatures except 16 °C vs. 18 °C, and the mean body size of the temperate females was consistently larger than the tropical females across the thermal range (FDR < 0.05, Fig. 2c). Egg-to-adult viability was less variable within populations; tropical flies were less viable at 16 °C compared to 22 °C, 25 °C and 28 °C and at 16 °C vs. 25 °C for the temperate population, whereas viability was higher in temperate flies at 18 °C vs. 30 °C and 25 °C vs. 30 °C (FDR < 0.05, Fig. 2d). There were no between-population differences at each temperature.

We also analysed thermal reaction norms using linear and nonlinear multiple regressions. Temperature had a significant positive linear relationship with heat knockdown time in both populations (Table S4), where knockdown resistance improved with increasing rearing temperature (Fig. 2a). Reaction norms for chill coma recovery in both populations were negative quadratic, with significant main effects of temperature and temperature^2 (Fig. 2b, Table S4). There was a strong negative linear relationship between temperature and body size, where size decreased with increasing rearing temperature (Fig. 2c, Table S4). Egg-to-adult viability reaction norms were quadratic, with the parabola concave downwards (i.e. 'bell' shaped, Fig. 2d), and although both temperature and temperature^2 main terms were significant, the overall model explained very little of the variation in viability (Table S4).

Effects of thermal regime and genotype on transcript-level phenotypes

Similar to the quantitative traits, rearing temperature had the most significant effect on mean transcript expression (22/23 transcript, three-way ANOVA, Table S5). Transcript abundance differed between the populations for 15/23 transcripts (Table S5), with a bias towards higher mean expression in tropical females (12/15 transcripts, Fig. 3). There was little evidence of G × E for expression variation; only two transcripts had significant temperature-by-population interaction terms (*Hsf-RA* and *mag*, $P < 0.05$ and 0.01, respectively, Table S5); however, these terms did not remain significant following FDR correction. For brevity, we restrict our results to description of reaction norms and not planned contrasts of means within and between populations.

Negative linear expression reaction norms in both populations

We observed an array of gene expression reaction norms fitting to first- to fourth-order polynomial functions (Table S3). For each transcript and population, linear and nonlinear multiple regressions were performed with the main terms fit after choosing the order of the reaction norm based on the BIC best-fitting

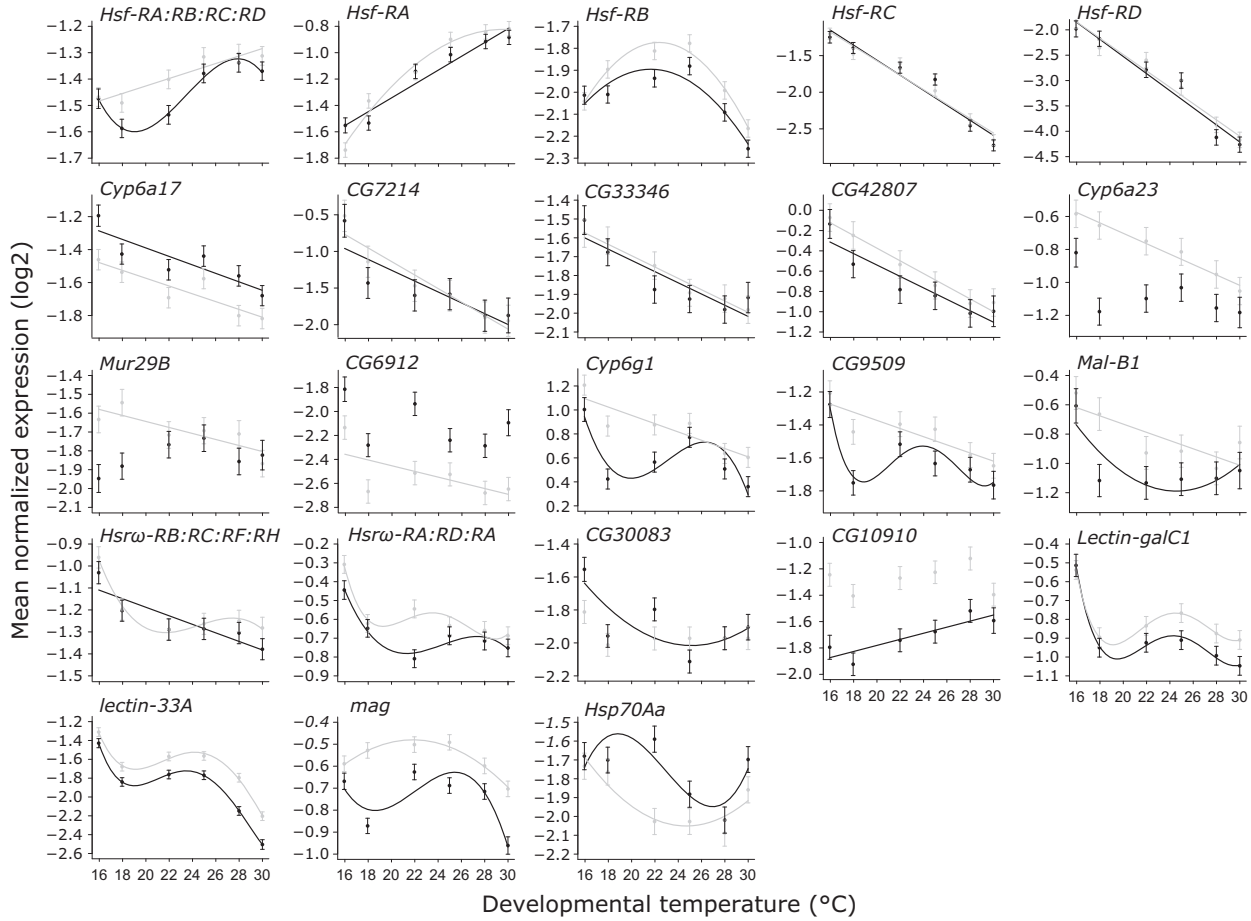

Fig. 3 Gene expression thermal reaction norms in temperate (black) and tropical (grey) flies for 23 mRNA transcripts (18 genes). Error bars represent standard error of the least-squares means.

models (Fig. 3; Table S6). For six transcripts, the main effect of temperature was significant in the linear regression in both the tropical and temperate populations (*Hsf-RC, Hsf-RD, Cyp6a17*, *CG7214*, *CG33346*, *CG42807*, Table S6, Fig. 3). The populations were invariant in trait means for the latter transcripts except *Cyp6a17* (ANOVA, Table S5), and the reaction norms were negative linear, that is expression decreased with increasing rearing temperature (Table S6, Fig. 3).

Population-specific, negative linear and nonlinear expression reaction norms

For three genes *Cyp6a23*, *Mur29B* and *CG6912*, the expression reaction norms were significantly negative linear in the tropical population only, whereas the main term of temperature was not significant in the temperate population (Table S6, Fig. 3). For three genes *Cyp6g1*, *CG9509* and *Mal-B1*, the reaction norms were negative linear in the tropical population and negative quadratic in the temperate population (Table S6, Fig. 3). Temperature had a significant quadratic relationship with expression (decreasing with slight U shape) in temperate females for *CG30083*, but no main terms were significant in the tropical population (Table S6, Fig. 3).

The shape of the curves was also differentiated between the two populations for the *Hsrω* transcript subsets; the *Hsrω-RB:RC:RF:RH* temperate population reaction norms were negative linear, whereas the tropical population curve was sigmoid (s-shaped, decreasing with increasing rearing temperature) with significant temperature, temperature^2 and temperature^3 main terms in the multiple regression (Table S6, Fig. 3). Both population reaction norms for *Hsω-RA:RD:RG* were negative sigmoid as for *Hsrω-RB:RC:RF:RH*, with an additional temperature^4 component in the multiple regression in the tropical population (Table S6, Fig. 3).

Population-specific positive linear and nonlinear expression reaction norms

Rearing temperature had a positive linear relationship with expression in both populations for *CG10910*, but

was specific to temperate flies for *Hsf-RA* and tropical flies for *Hsf-RA:RB:RC:RD* (Table S6, Fig. 3). The tropical population *Hsf-RA* reaction norm was better described by a quadratic rather than linear model (Table S6, Fig. 3). Temperate expression of *Hsf-RA:RB:RC:RD* was more complex than the tropical population where expression was lowest at 18 °C and 22 °C and highest at 25–30 °C, resulting in a positive s-shaped curve with significant linear, quadratic and cubic temperature main terms in the multiple regression (Table S6, Fig. 3).

Higher-order nonlinear expression reaction norms

For three transcripts, both populations exhibited similar complex thermal expression curves (*Hsf-RB*, *Lectin-galC1*, *lectin-33A*, Fig. 3). The *Hsf-RB* reaction norms were convex curvilinear (i.e. 'bell' shaped), whereas reaction norms for *Lectin-galC1* and *lectin-33A* were complex, quartic-shaped curves where expression was highest and lowest at the low and high temperature extremes, respectively, with intermediate expression in the mid-temperature range (Table S6, Fig. 3). We also observed population-specific, nonlinear reaction norms for two transcripts, *mag* and *Hsp70Aa*, where the tropical populations exhibited quadratic reaction norms whereas the temperate population reaction norms were higher-order cubic with significant temperature, temperature^2 and temperature^3 main terms in the multiple regression (Table S6, Fig. 3).

Isoform-specific expression reaction norms: Heat-shock factor (Hsf)

Interestingly, separate quantification of the four *Hsf* transcript isoforms revealed not only differences in the effects of thermal regime and population on expression means (ANOVA, Table S5), but also variation in reaction norms that differed from the 'gene-level' reaction norms (Fig. 3). Quantification of *Hsf* expression at the gene level (i.e. *Hsf-RA:RB:RC:RD*) would suggest that expression largely increases with rearing temperature with slightly different curve shapes between the populations, and whereas this is true for one isoform *Hsf-RA*, the *Hsf-RC* and *Hsf-RD* isoform curves were parallel negative linear, whereas for *Hsf-RB*, both populations exhibited bell-shaped reaction norms (Table S6, Fig. 3).

Cross-study comparisons of transcript thermal response means

Given our evidence-based approach in choosing a test set of loci for assessing thermal responses of transcript phenotypes in natural populations of *D. melanogaster* (see Materials and Methods), we next compared our data where possible to that in the literature. For the 18 genes examined here, we documented whether all main terms in the ANOVAS (temperature, population and temperature-by-population interactions) were tested in the other studies, which sex was assessed and genetic background of the populations (inbred or outbred), and where overlap between our study and other studies for a particular term/terms occurred (Table 3). As we undertook a candidate gene approach for 18 genes based on previous findings across a range of full transcriptome studies, we could not statistically quantify the degree of overlap but rather qualitatively report common outcomes (Table 3).

'Core' genes with thermal plasticity/and or geographic variation in diverse Drosophila melanogaster

For thermal plasticity, we observed a high degree of overlap with Chen *et al.* (2015) (15/18 genes, Table 3) and Levine *et al.* (2011) (13/18 genes, Table 3) and next with the outbred north American populations (Zhao *et al.*, 2015) (8/18 genes, Table 3). A core group of four thermally responsive genes were common to all studies: *Cyp6a23*, *Mal-B1*, *CG7214*, *CG42807* (Table 3). Thermal plasticity of heat-shock-related genes was observed across studies: *Hsrω* (here, Zhao *et al.*, 2015; and Chen *et al.*, 2015; Table 3), *Hsp70Aa* (here and Zhao *et al.*, 2015; Table 3) and *Hsf* (here and Chen *et al.*, 2015; Table 3).

Where we could compare differential expression of genes between populations, we found seven genes overlapped with the north American populations (Zhao *et al.*, 2015; Table 3). Apart from the current study and Levine *et al.* (2011), only Zhao *et al.* (2015) tested for G × E but they found no significant interactions for the 18 genes examined here (Table 3).

Comparison of gene expression reaction norms with other Drosophila melanogaster from the east coast of Australia

The populations studied here, and in Levine *et al.* (2011), were geographically most comparable: here, females from temperate and tropical populations (southern temperate Melbourne and northern tropical Innisfail) were reared at six temperatures, whereas Levine *et al.* (2011) compared males from a more southern temperate population (Tasmania) to tropical Innisfail reared at 18 °C and 30 °C. The latter temperatures were chosen with the rationale that they approximate the average 'home' temperatures naturally experienced by flies from the temperate and tropical populations, respectively (Levine *et al.*, 2011). The authors reported significant temperature effects on the transcriptome, G × E for 56 genes (FDR 0.1) as well as enrichment of 'home and away' directionality of expression (i.e. higher expression in temperate flies reared at 18 °C vs. attenuated expression at 30 °C and *vice versa* in the tropical population).

We examined 12 genes exhibiting G × E in Levine *et al.* (2011), and although temperature significantly impacted all genes and 10 genes were differentially expressed between the populations (Table 3), we found little evidence for G × E or 'home and away' gene

Table 3 Summary of the cross-study comparison for the 18 *Drosophila melanogaster* genes chosen in the current study based on previous evidence of temperature expression plasticity[1,2,3], and/or genotype (geographic) expression variation[1,2] and/or genotype-by-environment interactions (G × E) between population of origin and thermal regime[1,2,3]. Significant genes* in each study are shown for each category (N/A denotes where terms were not assessed in a study).

Study/design	Thermal plasticity	Genotype/geographic variation	G × E	Inbred (I) or Outbred (O)	Sex
Current study EA Cline ends 16 °C, 18 °C, 22 °C 25 °C, 28 °C, 30 °C	*Hsf*, *Hsrω*, *Hsp70Aa*, *Cyp6a17*, *Cyp6a23*, *Cyp6g1*, *Lectin-galC1*, *lectin33A*, *mag*, *Mal-B1*, *CG6912*, *CG7214*, *CG9509*, *CG10910*, *CG30083*, *CG33346*, *CG42807*	*Hsf*, *Hsrω*, *Hsp70Aa*, *Cyp6a17*, *Cyp6a23*, *Cyp6g1*, *Lectin-galC1*, *lectin33A*, *mag*, *Mal-B1*, *Mur29B*, *CG6912*, *CG9509*, *CG10910*, *CG30083*	*Cyp6a23*†, *mag*†, *Mur29B*†	O	Female
Zhao *et al.* (2015)[1] NA Cline ends 21 °C, 29 °C	*Hsrω*, *Hsp70Aa*, *Cyp6a23*, *Lectin-galC1*, *Mal-B1*, *CG7214*, *CG9509*, *CG42807*	*Cyp6a23*, *Cyp6g1*, *Lectin-galC1*, *Mur29B*, *CG6912*, *CG9509*, *CG42807*	None	O	Male
Levine *et al.* (2011)[2] EA Cline ends 18 °C, 30 °C	*Cyp6a17*, *Cyp6a23*, *Lectin-galC1*, *lectin-33A*, *mag*, *Mal-B1*, *Mur29B*, *CG6912*, *CG7214*, *CG10910*, *CG30083*, *CG33346*, *CG42807*	N/A	*Cyp6a17*, *Cyp6a23*, *Lectin-galC1*, *lectin-33A*, *mag*, *Mal-B1*, *Mur29B*, *CG6912*, *CG10910*, *CG30083*, *CG33346*, *CG42807*	O	Male
Chen *et al.* (2015)[3] Lab strains 13 °C, 18 °C, 23 °C, 29 °C	*Hsf*, *Hsrω*, *Cyp6a17*, *Cyp6a23*, *Cyp6g1*, *lectin33A*, *mag*, *Mal-B1*, *CG6912*, *CG7214*, *CG9509*, *CG10910*, *CG30083*, *CG33346*, *CG42807*	N/A	N/A	I	Female

EA, eastern Australia; NA, north America.

*Significance thresholds vary from study to study; we used genes from reported results.

†Significant interaction terms in the ANOVA in the current study that were nonsignificant at FDR 0.05.

expression directionality across six rearing temperatures apart from weak signal at the *mag* locus (ANOVA, Table S5). For a more direct comparison, we next analysed trait means only for 18 °C and 30 °C using three-way ANOVAs. We found less thermal plasticity at 18 °C and 30 °C compared to Levine *et al.* (2011) than across our full thermal range, with only five genes significant for the main effect of temperature (Table S7, Fig. S3). The populations differed in transcript abundance for seven genes, biased to higher expression in the tropical population (Table S7, Fig. S3). Only two genes, *Cyp6a23* and *Mur29B*, had significant gene-by-environment interaction terms, although the direction of expression was opposite to the 'home and away' pattern observed by Levine *et al.* (2011) and similar to *mag* for all six temperatures, although the significance was lost after FDR correction (uncorrected $P < 0.05$, FDR < 0.2, Table S7).

Comparison of gene expression reaction norms with inbred Drosophila melanogaster

Chen *et al.* (2015) classified gene expression reaction norms in inbred female *D. melanogaster* across a broader thermal regime overlapping with the range employed here (ranging from 13 °C to 29 °C). Where possible, we compared the direction of expression plasticity (i.e. increasing or decreasing with temperature) and reaction norm curvature (Table 4). Fifteen genes were comparable between the two studies (Tables 3 and 4); there was concordance for the direction of expression plasticity (10/15 genes, Table 4), with 8/10 genes decreasing in expression with increasing rearing temperature, one gene increasing with rearing temperature and one gene with *U*-shaped expression over the thermal regimes (Table 4). There was also overlap in reaction norm shape (7/15 genes, Table 4) where at least one of the populations here exhibited the same curvature as Chen *et al.* (2015).

Discussion

We compared thermal plasticity between female *D. melanogaster* from the ends of the eastern Australian temperate-tropical latitudinal gradient for 28 phenotypes across six rearing temperatures. Temperature impacted almost every phenotype ranging from quantitative fitness and morphological traits to gene transcripts. Although there were some differences between the populations for quantitative trait means, fecundity thermal performance and reaction norms for thermotolerance, body size and viability were comparable, supporting a view of ectotherm thermal adaptation by shifts in average trait values rather than reaction norm shape (Yamahira *et al.*, 2007; Klepsatel *et al.*, 2013a;

Fragata *et al.*, 2015). The gene expression traits revealed more complexity in response curves between the populations, although we found little evidence for a genetic component underpinning the plasticity variation in contrast to previous findings (Levine *et al.*, 2011).

Similar performance and reaction norm variation in quantitative traits

For reproductive performance measured as absolute fecundity, we found no difference between tropical and temperate thermal optima or performance breadth, in agreement with cross-continent *D. melanogaster* populations (Klepsatel *et al.*, 2013a). The temperate females had higher maximum output, however, consistent with previous *Drosophila* studies which reject the 'hotter is better' hypothesis of performance (Klepsatel *et al.*, 2013a; Fragata *et al.*, 2015). 'Hotter is better' predicts a positive correlation in maximal performance with increased thermal selection, that is higher output from the tropical population (Angilletta *et al.*, 2010). Higher output of temperate flies could result from the positive correlation with body size and fecundity (see Klepsatel *et al.*, 2013b), but we failed to find a relationship despite the larger size of temperate females across the thermal range. Unlike some *D. subobscura* populations, 'bigger wasn't always better' (Fragata *et al.*, 2015); we observed no differences at the highest temperature (30 °C), and the tropical females were more fecund than temperate females at the mildest temperature (16 °C). The latter result is surprising given that larger more cold-adapted flies often perform better at lower temperatures (Reeve *et al.*, 2000; Bochdanovits & De Jong, 2003).

In contrast to our results, Klepsatel *et al.* (2013a) found higher fecundity in temperate *D. melanogaster* at intermediate temperatures whereas tropical females performed better at high temperatures, and in another study measuring reproductive output as productivity, temperate populations did better in the cold but worse in the heat compared to tropical populations (Trotta *et al.*, 2006). It is unclear what factors underlie the differences; here, possible explanations include insufficient power to detect differences using a narrower, constant thermal range in two populations from the same climatic gradient compared to the six cross-continent populations reared under the fluctuating regime employed by Klepsatel *et al.* (2013a). On the Australian east coast, although average minimum temperatures decrease with latitude, it is possible that thermal selection at upper temperatures experienced by the populations studied here may be similar given maximum yearly temperatures are largely uniform, and maximum summer temperatures are similar between tropical Innisfail and temperate Melbourne (Hoffmann, 2010; Overgaard *et al.*, 2011; Kristensen *et al.*, 2015). Average temperatures, however, do not reflect sudden fluctuations in temperature extremes, which are experienced more frequently by temperate eastern Australian flies compared to their tropical counterparts (Hoffmann, 2010). Despite evidence for thermal selection on the Australian east coast in opposing thermotolerance clines (Hoffmann *et al.*, 2002; Sgrò *et al.*, 2010), we found little variation between the populations for chill coma recovery and heat knockdown; rather, the temperate population better resisted heat knockdown at 16 °C and 18 °C and recovered from cold exposure faster at 30 °C. Maintaining higher stress resistance at the ends of the thermal range could reflect a better ability of temperate flies to withstand temperature extremes. In the laboratory, temperate Australian *D. melanogaster* may be physiologically more capable to withstand sudden, extreme

Table 4 Cross-study comparison of *Drosophila melanogaster* gene expression reaction norms for 15 genes assessed here (reared at 16 °C, 18 °C, 22 °C 25 °C, 28 °C, 30 °C) with Chen *et al.* (2015) (reared at 13 °C, 18 °C, 23 °C, 29 °C).

	Curvature (current study)				
Gene	Tropical	Temperate	Curvature (Chen *et al.*, 2015)	Plasticity (current study)	Plasticity (Chen *et al.*, 2015)
Hsf	Linear	Quadratic	Linear	Increasing	Increasing
CG33346	Linear	Linear	Linear	Decreasing	Decreasing
Cyp6a17	Linear	Linear	Quadratic	Decreasing	Decreasing
CG7214	Linear	Linear	Quadratic	Decreasing	Decreasing
CG42807	Linear	Linear	Quadratic	Decreasing	Decreasing
Cypg1	Linear	Quadratic	Linear	Decreasing	Decreasing
CG9509	Linear	Quadratic	Linear	Decreasing	Decreasing
Mal-B1	Linear	Quadratic	Linear	Decreasing	Decreasing
CG6912	Linear	–	Quadratic	Decreasing	Decreasing
CG30083	–	Quadratic	Quadratic	U	U
Cyp6a23	Linear	–	Quadratic	Decreasing	Bell
Mur29B	Linear	–	Quadratic	Decreasing	U
CG10910	Linear	Linear	Quadratic	Increasing	U
Lectin-33A	Quartic	Quartic	Linear	Decreasing	Increasing
Mag	Quadratic	Cubic	Quadratic	Decreasing	Bell

temperature changes than tropical populations (Sgrò *et al.*, 2010), although the extent to which natural extremes are mitigated via behavioural avoidance through habitat selection could be important (Feder *et al.*, 2000).

Despite variations in mean thermotolerance between the populations, the reaction norms were parallel in shape, consistent with observations of similar thermotolerance plasticity between *D. melanogaster* populations (Hoffmann & Watson, 1993; Bubliy *et al.*, 2002; Hoffmann *et al.*, 2005; Cooper *et al.*, 2012) and among widespread and tropical *Drosophila* species (Overgaard *et al.*, 2011). We found that rearing temperature impacted the traits in the direction anticipated, that is increased resistance to heat knockdown with increasing rearing temperature and the opposite for chill coma resistance, consistent with high levels of plasticity for stress resistance in response to environmental conditions (discussed in Hoffmann *et al.*, 2005). The similarity in phenotypic plasticity at the intra- and interspecific level holds independent of thermal regimes (i.e. developmental or short-term acclimation, fluctuating or constant conditions).

For body size, the plastic (i.e. decrease in size with increasing temperature) and genetic responses (consistently larger temperate females) both comprised parallel vertical shifts in the trait means, consistent with most intraspecific comparisons, including continent-wide clinal studies and/or reaction norm analyses (Coyne & Beecham, 1987; James *et al.*, 1995; Land *et al.*, 1999; Trotta *et al.*, 2006), but see Morin *et al.* (1999). We found no population differences in reaction norms for viability, a key indicator of pre-adult fitness, in agreement with European *D. melanogaster* and *D. simulans* populations (Petavy *et al.*, 2001). Similarly, thermal plasticity for viability to alternating regimes was invariant between highland vs. lowland Argentinian populations sampled from opposing latitudinal and altitudinal viability clines (Folguera *et al.*, 2008), in contrast, however, to South American populations that differed slightly in reaction norms, whereas trait values did not vary over latitude (Land *et al.*, 1999). Here, however, the impact of temperature was mild, and viability remained above 80% across the thermal range, similar to observations between 14 and 28 °C in *D. melanogaster* and *D. simulans* (Petavy *et al.*, 2001). Therefore, we did not assess viability performance as a trait given our thermal range did not quite capture the stressful temperatures (particularly at the low end) (Petavy *et al.*, 2001; Kristensen *et al.*, 2015) that might more clearly define performance parameters such as performance breadth.

Expression plasticity

The high degree of thermal plasticity at the transcript level (22/23 transcripts) is unsurprising given our gene selection criteria, with the majority also thermally responsive among different *D. melanogaster* transcriptome studies (Levine *et al.*, 2011; Chen *et al.*, 2015; Zhao *et al.*, 2015). We found four 'core' genes with consistent temperature modulated expression independent of genetic background or study design, suggesting some degree of conserved thermal plasticity in *D. melanogaster*. Chen *et al.* (2015) reported a higher degree of thermal plasticity than previous estimates from fewer temperatures (Levine *et al.*, 2011; Zhou *et al.*, 2012; Zhao *et al.*, 2015), and for our gene set, we observed the most overlap with Chen *et al.* (2015) likely due to the broader thermal regimes employed, where plasticity increases with additional environmental exposures.

Although not as extensive as the effect of temperature, mean expression of two-thirds of the genes differed between the tropical and temperate populations, with higher expression levels predominantly biased to the tropical population. Expression variation within and between populations is well documented (Oleksiak *et al.*, 2002; Michalak *et al.*, 2007; Levine *et al.*, 2011; Müller *et al.*, 2011; Catalán *et al.*, 2012), reflective of ample genetic variation for differential transcript abundance, although the fitness consequences of gene expression divergence remain largely unknown (Feder & Walser, 2005; Evans, 2015). Expression differences often arise from *cis* regulatory variation and also copy number variation: here two differentially expressed examples are *Cypa17* and *Cyp6g1*, confirming previous work (Hutter *et al.*, 2008; Catalán *et al.*, 2012) and copy number variation for these genes are known and related to expression for DDT-like pesticide resistance (Schmidt *et al.*, 2010) which may vary according latitude along the east Australian coast (Turner *et al.*, 2008). Despite the lack of evidence here, there is increasing support for a role of spatially varying selection in maintaining adaptive gene expression variation in diverse environments, where differences in adaptive phenotypes may be evidenced through plasticity (see Levine *et al.*, 2011). G × E interactions maintain variation in plasticity across different genotypes and are prevalent in gene expression data (Levine *et al.*, 2011; Dayan *et al.*, 2015; Zhao *et al.*, 2015), although as above, the contribution of gene expression plasticity to organismal fitness is less clear (Hodgins-Davis & Townsend, 2009), but general patterns can provide broader insight into evolution in heterogeneous environments.

Lack of support for G × E for expression plasticity

We expanded the developmental thermal regime for 12 highly significant genes from previous research reporting expression G × E at 18 °C and 30 °C in eastern Australian *D. melanogaster* (Levine *et al.*, 2011), but failed to replicate G × E or directional 'home and away' expression plasticity of expression trait means, even when compared only 18 °C and 30 °C. One

explanation is study design differences; Levine *et al.* (2011) pooled males from isofemale lines whereas we assessed mass-bred females derived from isofemale lines which may impact gene expression comparisons owing to patterns of linkage disequilibrium and/or sex-specific effects. Further, although the tropical populations were from the same town in both studies, we assessed a temperate southern mainland population whereas Levine *et al.* (2011) compared a temperate population further south from island Tasmania, although Tasmanian *D. melanogaster* are not isolated given the evidence for gene flow between populations (Kennington *et al.*, 2003).

Standing genetic variation in different populations will impact G × E; genes that exhibit differential expression for genotypes in different environments are impacted by local but predominantly upstream regulatory sequence variation (Hodgins-Davis & Townsend, 2009; Grishkevich & Yanai, 2013) and at the genome-wide level may even be categorized as more likely to exhibit G × E by distinctive genomic and structural features (Grishkevich & Yanai, 2013). As such, differences in DNA polymorphisms in the temperate populations examined here, and by Levine *et al.* (2011), compared to tropical Innisfail could be a factor, and we also cannot rule out the impact of seasonal variation on standing genetic variation given the extensive temporal shifts documented in natural *D. melanogaster* (Itoh *et al.*, 2010; Bergland *et al.*, 2014). Perhaps, the highly environmentally plastic nature of the transcriptome (Hodgins-Davis & Townsend, 2009) coupled with genetic shifts from sampling season renders replicable signatures of G × E for gene expression traits difficult between temperate and tropical eastern Australian populations.

Although there was little support for genetic variation in mean expression plasticity between the populations, there were differences in the thermal expression reaction norm curves, suggesting a high degree of population-specific plastic variation in contrast to the largely parallel quantitative trait reaction norms. Differences in the shape of the response curves between populations could be passive responses due to neutral sequence variation, thermal stress or other unknown constraints (Gibert *et al.*, 1998; Levine *et al.*, 2011), or they could be adaptive and therefore useful in identifying putative selection targets (Gibert *et al.*, 1998). The results are promising for future work harnessing the transcriptome as a powerful set of traits and broader thermal regimes to explore the evolutionary basis of plasticity in a modelling framework (e.g. Gibert *et al.*, 1998). Although the reaction norms were surprisingly dissimilar with previous eastern Australian *D. melanogaster* (Levine *et al.*, 2011), we found repeatability in the direction of plasticity and curvature in at least one of our populations with Chen *et al.* (2015), highlighting common expression responses to a broader thermal range, but whether the same mechanisms underpin the trait responses remains an open question. It is worth noting that three of the five genes (*Cyp6a23*, *mag* and *Mur29B*) that exhibited different reaction norms between the populations here and also compared to Chen *et al.* (2015) exhibited weak evidence for G × E in the current study (either across the entire thermal range or at 18 °C and 30 °C), suggestive of segregating genetic variation for plasticity in these genes, although more population data are required to further explore this.

Further complexity of transcript reaction norm variation: *Hsf*

Finally, in addition to testing genes with previous population-level expression differentiation and/or population-by-temperature interactions, we examined plasticity in three key genes involved in the heat-shock response, *Hsp70Aa*, *Hsrω* and the master regulator *Heat-shock factor* (*Hsf*). Chen *et al.* (2015) also reported reaction norms for a number of *Hsps,* and similar to here, the genes do not always show a clear relationship with temperature for this thermal range. Although we found differences in thermal regulation of the genes, the thermal plasticity appears not to have a discernible genetic component, and in combination with the largely similar thermotolerance phenotypes, data suggest a lack of divergence in these populations. Linking heat-shock genes to adaptive thermotolerance is problematic, however (Telonis-Scott *et al.*, 2014), but what was striking here was the complexity of reaction norms for the isoforms of *Hsf* compared to the gene-level reaction norms. Previously, Fujikake *et al.* (2005) identified alternative isoforms of *Hsf* with two isoforms, *B* and *D* differentially elicited under heat and cold stress, respectively, suggesting that in addition to post-transcriptional modifications, transcription of the gene is autoregulated during thermal stress via alternative splicing. We examined the four isoforms *RA-D* and report three different reaction norms including increasing (*RA*), bell shaped (*RB*) and decreasing (*RC* and *RD*). This speaks of the complexity of the locus but also highlights the complex nature of transcript-level phenotypes where gene isoforms may present as separate traits. Understanding the evolution of traits ultimately depends on how traits are measured here for a gene and also for quantitative traits; for instance, the reaction norms for the final traits of insect size and growth revealed less genetic variation for plasticity than thermal performance curves for growth rate (Kingsolver *et al.*, 2004).

Conclusion

In conclusion, we found that populations from tropical and temperate east Australia exhibit similar thermal plasticity for quantitative traits despite evidence for

genetic variation for trait values. Our data therefore do not support a model of thermal evolution by plastic shifts but rather in overall trait means. We found no evidence for 'hotter is better' for performance; rather, an overall better performance of the temperate population was observed. Our study also incorporated an expanded trait set including a subset of genes exhibiting expression G × E from transcriptome studies, and whereas there was a higher degree of thermal plasticity for transcript traits, we found little support for a role of genetic variation in maintaining expression plasticity. Instead, we found most overlap in reaction norm shape for expression traits with another study with a similar thermal regime in contrast to studies using fewer exposures. This highlights the need to adequately sample thermal environments when examining the relative contribution of plasticity vs. trait mean divergence in populations. Further, the additional complexity in reaction norms between distinct isoforms of the *Hsf* gene demonstrates the importance of trait definition when inferring patterns of plastic and evolutionary responses.

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Supporting information

Additional Supporting Information may be found online in the supporting information tab for this article:
Figure S1 Wing vein landmark points used to determine wing centroid size. For details see Materials and Methods.
Figure S2 Thermal developmental reaction norms for temperate (black) and tropical (grey) flies developed at 18 °C and 30 °C for the twelve transcripts common to this study and Levine *et al.* (2011).
Table S1 Number of generations that each population (Te = Temperate; Tr = Tropical) was maintained in the laboratory at 25 °C before each assay was performed.
Table S2 Primer sequences used for real-time PCR. *Hsf* transcript sequences taken from Fujikake *et al.* (2005).

Table S3 Bayesian Information Criterion (BIC) values for functions fitted to heat tolerance, cold resistance, egg-to-adult viability and transcript abundance data.
Table S4 Linear regression (heat resistance and body size) and multiple non-linear regression (Cold resistance and egg-to-adult viability) results for the quantitative traits.
Table S5 Three-way fixed-effects general linear-model ANOVA results for transcript abundance for the effects of developmental temperature, population (temperate vs. tropical), and the interaction between them*.
Table S6 Multiple linear and non-linear regression for transcript abundance data.
Table S7 Three-way fixed-effects general linear-model ANOVA results for transcript abundance showing the effects of developmental temperature, population, and the interaction between them for temperate and tropical flies developed at 18 °C and 30 °C for the twelve transcripts common to this study and Levine *et al.* (2011).

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