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ORIGINAL ARTICLE

Latitudinal and cold-tolerance variation associate with DNA repeat-number variation in the *hsr-omega* RNA gene of *Drosophila melanogaster*

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An 8-bp deletion in the *hsr-omega* heat-stress gene of *Drosophila melanogaster* has previously been associated with latitude, and with heat tolerance that decreases with latitude. Here we report a second polymorphic site, at the 3′-end of *hsr-omega*, at which multiple alleles segregate in natural populations for copy number of a ~280 bp tandem repeat. On each of 3 consecutive years (2000, 2001 and 2002) among populations sampled along the Australian eastern coast, repeat number was negatively associated with latitude. Neither altitudinal association was detected in 2002 when five high-altitude sites were included, nor was a robust association detected with local temperature or rainfall measures. Although in a large number of family lines, derived from a population located centrally in the latitudinal transect, no association between *hsr-omega* repeat number and heat tolerance occurred, a negative association of repeat number with cold tolerance was detected. As cold tolerance also exhibits latitudinal clines we examined a set of cold-tolerant populations derived by selection and found both reduced repeat number and low constitutive levels of the *omega-n* repeat-bearing transcript. In a sample from the central population, linkage disequilibrium was measured between repeat number and linked markers that also cline latitudinally. However, such disequilibrium could not account for the cline in repeat number or tolerance associations. Finally, during adult recovery from cold exposure a large increase occurred in tissue levels of the *omega-c* transcript. Together these data suggest that a latitudinal cline in *hsr-omega* repeat number influences cold-tolerance variation in this species.
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Introduction

Physiological and genetic mechanisms that underpin thermal tolerance variation among species and among ecotypes of the same species have long been a topic of interest (Cossins and Bowler, 1987; Hoffmann and Parsons, 1991; Zatsepina *et al*., 2000) especially now that we have a heightened awareness of the threats to animal and plant populations under global warming (Lovejoy and Hanna, 2006). *Drosophila* species have been of considerable focus in attempts to elucidate such mechanisms because, apart from the obvious benefits of their experimental and genomic tractability, there are numerous examples of closely related species with quite different thermal tolerance attributes (Hoffmann *et al*., 2003) and of intra-specific ecotype variation where strains from warm tropical regions at low latitudes are more heat tolerant and/or more cold sensitive than those from cool temperate higher latitudes (Kimura *et al*., 1994; Guerra *et al*., 1997; Hoffmann *et al*., 2002). Heritable, climatically-associated variation in stress resistance traits provide the opportunity to investigate and understand the potential of and limits to the processes of adaptation to thermal extremes.

A large number of genes have been identified that are helping to elucidate cellular and physiological mechanisms that protect from heat stress (Hoffmann *et al*., 2003; Sørensen *et al*., 2005). Multiple processes are clearly involved and these need to be identified and understood. Of the candidate genes that have been the subject of more intensive investigation, the best characterized, especially in *Drosophila*, is the *hsp70* gene (see Bettencourt *et al*., 2002 and Gong and Golic, 2006, and references therein). Although it is generally recognized that *hsp70* is an essential component of the cellular heat-stress response, and that in general high levels of expression of *hsp70* are more protective, some measures of heat resistance show a negative association with expression of *hsp70* (Zatsepina *et al*., 2001; and see discussion in Hoffmann *et al*., 2003 and Chen *et al*., 2008). Other reports implicate the importance of different heat-shock protein genes (McColl *et al*., 1996) and novel genes (Ekengren and Hulmark, 2001) for thermal tolerance variation.

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In *Drosophila melanogaster* one candidate gene that has been subject to thorough investigation at the molecular and cellular level is *hsr-omega* (Pardue *et al*., 1990). This gene does not produce a protein product. It produces two main RNA transcripts both of which are constitutively expressed in most adult tissue types, one located in the cytoplasm, *omega-c*, and one in the nucleus, *omega-n*. *hsr-Omega* has long been recognized as one of the heat-stress genes as it is upregulated by a mild heat shock (similar to the heat-shock protein genes), and because overlapping deletion mutants of *hsr-omega* are thermosensitive (Pardue *et al*., 1990). Also, *hsr-omega* shares with the other heat-shock genes isolated from natural populations an abundance of *P*-element inserts in the promoter region (Walser *et al*., 2006)—inserts that may influence heat-shock gene expression and thermal adaptive potential (Chen *et al*., 2008).

Further, in parallel with clines in thermal tolerance along a latitudinal gradient on the eastern coast of Australia, an 8-bp indel polymorphism in the *hsr-omega* gene (the *hsr-omega-L/S* indel polymorphism; Figure 1) shows a strong frequency cline that has been associated with average maximum temperatures of the hottest month (Anderson *et al*., 2003). The shorter allele (*hsr-omega-S*), characterized by the 8-bp deletion in the first exon (80 bp upstream of the intron 5′ splice site; Figure 1; McKechnie *et al*., 1998), occurs at high frequency in Australian tropical populations, and is strongly and negatively associated with latitude (Anderson *et al*., 2003). Furthermore, both *hsr-omega-S* allele and *hsr-omega* transcript levels have been associated with heat-stress resistance in selected lines (McColl *et al*., 1996; McKechnie *et al*., 1998).

Various measures of heat resistance have been used in recent studies, some of which have found associations with *hsr-omega-L/S* variation and some of which have not (Weeks *et al*., 2002; Norry *et al*., 2004; Morgan and Mackay, 2006; Rako *et al*., 2007). One of the confounding factors in these association studies is the linkage disequilibrium of *hsr-omega* with breakpoints of the common cosmopolitan inversion *In(3R)Payne* (Kennington *et al*., 2006). The *hsr-omega* gene is cytologically located within this major cosmopolitan inversion, and the *hsr-omega-S* allele is strongly associated with its presence (Anderson *et al*., 2005a). Generally, inversions are known to maintain certain allele combinations across loci by suppressed recombination, thus in this instance any genotype/resistance association only provides weak evidence of a causal role for *hsr-omega*.

What we know about the cellular biology of the *hsr-omega* transcripts however is consistent with it having a role in the heat-stress response that has been well characterized in *Drosophila* (Lindquist, 1980). Although the heat-stress response involves many genes (Sørensen *et al*., 2005), a major component is the upregulation of heat-shock protein synthesis and downregulation of normal protein synthesis. Under heat stress, the *hsr-omega* transcripts are thought to play a role related to the integration of cytoplasmic and nuclear processes influencing protein synthesis (Pardue *et al*., 1990; Lakhotia, 2003). *hsr-Omega* is likely to be an important gene as its unusual structure and several small segments of its sequence are conserved across diverse *Drosophila* species. In addition, chromosome puffing at its cytological location following removal from cold rearing suggests that it may also play a part in recovery from cold exposure (Lakhotia and Singh, 1985). Finally, a unique and intriguing feature of the nuclear-located *omega-n* is a conspicuous polymorphism of a cluster of tandem repeats encoded at the 3′-end of the gene (Figure 1).

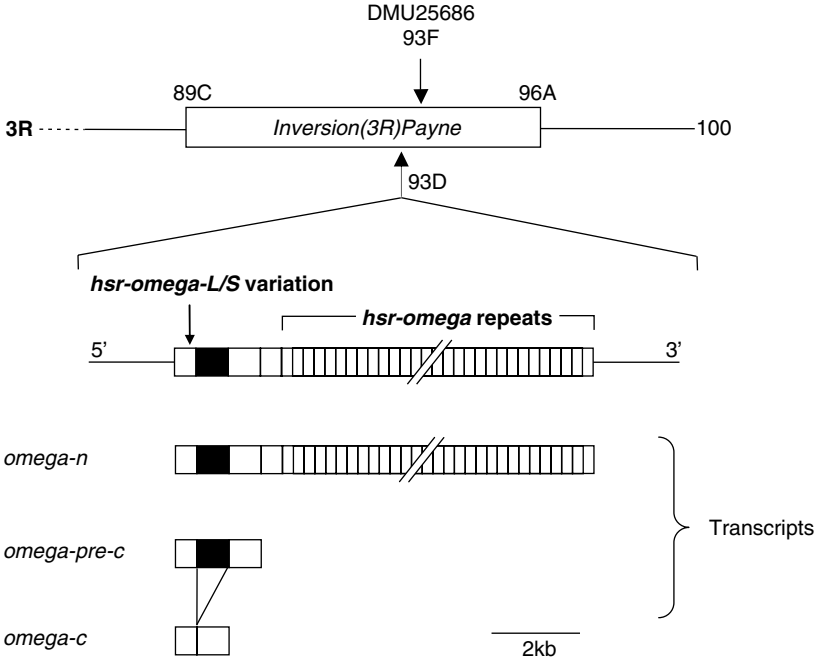

Figure 1 Right arm of chromosome 3 (3R) showing cytological location of the *hsr-omega* gene (93D), *In(3R)Payne* and microsatellite marker *DMU25686*, together with a map of *hsr-omega* depicting the relative positions of the 5′ L/S polymorphic sites, the intron (black box) and the 3′ repeat variation site. The three known *hsr-omega* transcripts, including the nuclear located *omega-n* and cytoplasmic located *omega-c*, are also shown.

The number of 280-bp repeat units, that constitute most of the *omega-n* transcript length, varies markedly among laboratory strains, producing alleles with repeat clusters between 5 and 16 kb in length (Walldorf *et al*., 1984; Hogan *et al*., 1995).

In this report we investigate aspects of naturally occurring variation in the *D. melanogaster hsr-omega* gene. Specifically, we report on latitudinal and altitudinal *hsr-omega* repeat-number variation along a climatic gradient on the eastern coast of Australia. We assess repeat-number association with the common cosmopolitan inversion *In(3R)P* and with the *hsr-omega-L/S* variation, in a population located at a central geographic location along the latitudinal gradient. We also look for associations of repeat number with thermal resistance traits in both a large set of family lines and a set of cold selected lines. As our data suggest an association between *hsr-omega* repeat number and both latitude and cold tolerance, we use a set of laboratory populations to examine changes in *hsr-omega* expression patterns following cold exposure. Our results support the hypothesis that variation in repeat number of *hsr-omega* is an important determining component of latitudinal variation in cold tolerance.

Methods

Field samples

Isofemale lines of *D. melanogaster* were established from field-caught females collected from banana bait trapping. Low-altitude isofemale lines were collected at regular intervals along the eastern coast of Australia spanning more than 26° (~3000 km) in February–March of 2000, 2001 and 2002. Five high-altitude collections were made in 2002, each at a similar latitude to a different low-altitude site. The location of collection sites and the number of isofemale lines sampled per site are indicated in Table 1 and Figure 2.

Quantifying *omega* repeat variation

Tandem repeat variation was genotyped by Southern blot. DNA extractions were carried out on batches of 25 flies (mixed sexes) by mortar–pestle grinding in 500 µl grinding buffer (0.2 M sucrose, 0.1 M Tris pH 9.2, 50 mM EDTA, 0.5% SDS), followed by the addition of 2 µl of proteinase K (20 mg ml^{-1}) and incubation for 10 min at 45 °C. After addition of 150 µl 8 M potassium acetate the extract was vortexed and placed on ice for 15 min, removed and 500 µl phenol/chloroform/isoamyl alcohol (25:24:1) added and re-vortexed for 30 s prior to 5 min microfuge centrifugation at 12 000 r.p.m. The supernatant was transferred to 1 ml of 100% ethanol, vortexed and put for 4 h at room temperature to precipitate the DNA. Following 10 min centrifugation at 12 000 r.p.m. the supernatant was discarded, the pellet gently washed with 70% ethanol and air dried prior to dissolving in MilliQ water overnight at 4 °C. In the 2000 and 2001 collection, extractions were performed on each isofemale line. In the 2002 collection, one extraction was performed per latitudinal collection site, where each extraction consisted of five isofemale lines pooled in equal proportions (five flies per isofemale line, sexes mixed). Prior to electrophoresis DNA (3.5 µg µl^{-1}) was double digested overnight at 37 °C with Pst*I* and Hin*d III* (Promega, Annandale, New South Wales, Australia) in buffer B according to manufacture's protocols (thus cleaving each end of the tandem repeat region; Hogan *et al*., 1995). Southern blot and hybridization were performed largely as described by Sambrook *et al*. (1989). The DNA was transferred to a Zeta-probe membrane (Bio-Rad, Gladesville, New South Wales, Australia) and probed with γ-^{32}P radio-labelled *D. melanogaster* repeat units. The radioactive membrane was exposed to X-ray film and developed in an automated X-ray machine.

As there were a large number of different band positions (alleles), some of which were difficult to distinguish, they were analysed in the 2000 collection by placing alleles into five different groups based on size. A size standard, containing four different repeat-length alleles spaced apart at 20.3, 14.5, 10.6 and 8.7 kb (created by pooling DNA from two isofemale lines) was electrophoresed on all gels in at least two well positions (Figure 3a). The size of the four fragments in the standard

Table 1 Collection sites for 2000–2002, showing the number of isofemale lines sampled for repeat-length variation each year

Location	*Latitude (S)*	*Altitude (m)*	*No. isofemale lines sampled*		
			2000	*2001*	*2002*
Cooktown	15°22′	<100		1	
Cape Tribulations	16°01′	<100		5	5
Mossman	16°27′	<100	8	2	
Malanda	17°19′	800			69
Innisfail	17°30′	<100	11	3	74
Townsville	19°15′	<100		14	5
Bowen	20°01′	<100	7		
Sarina	21°25′	<100	8	5	5
Rockhampton	23°22′	<100	9	3	5
Gladstone	23°50′	<100			5
Miriam vale	24°19′	<100	6		5
Maryborough	25°31′	<100	11		5
Rainbow Beach	25°54′	<100	2		5
Redland bay	27°39′	<100	7		5
Springbrook	28°14′	1031			17
Kingscliff	28°17′	<100		9	51
Duranbah	28°19′	<100	7		
Alstonville	28°50′	<100	3		5
Coffs harbour	30°19′	<100	11	11	58
Armidale	30°31′	1296			67
Port Macquarie	31°25′	<100			5
Belmont	33°02′	<100			5
Gosford	33°26′	<100	7		
Thirlmere	34°11′	<100		2	
Blackheath	33°38′	1046			48
Wollongong	34°25′	<100			53
Adaminaby	35°59′	1025			51
Cobargo	36°22′	<100		14	
Bega	36°39′	<100	13		41
Yan yean	37°34′	<100	6		
Wandin	37°47′	<100	4	5	
Coronet bay	38°26′	<100			5
Pomona	41°06′	<100		4	
Tamar valley	41°11′	<100	9	3	5
Windara	41°14′	<100		6	
Dilston	41°19′	<100			5
Red Knight	42°45′	<100			5
Huonville	43°02′	<100	8		
Scenic Road	43°02′	<100	5		
Franklin	43°05′	<100		9	
Panorama	43°07′	<100		10	

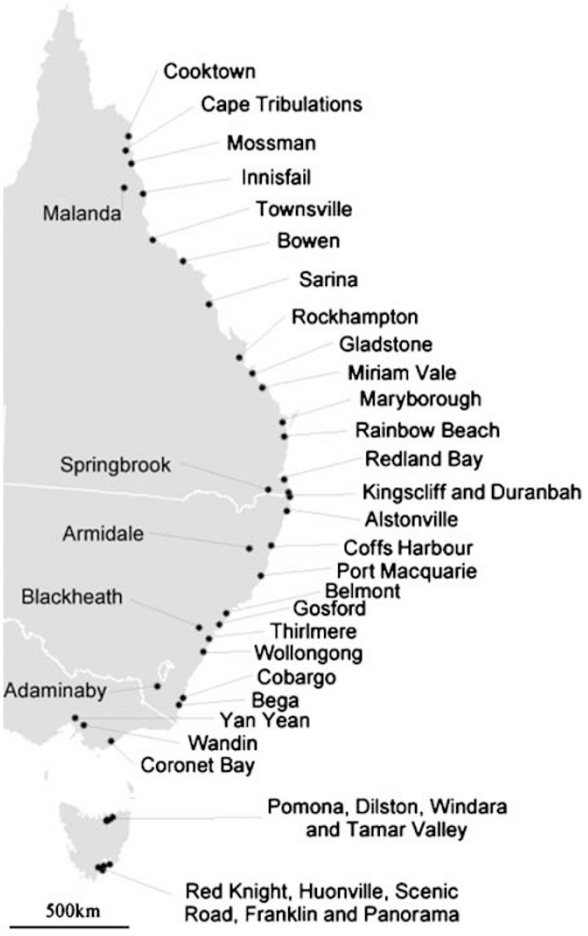

Figure 2 Collection sites for the latitude and altitude survey of *hsr-omega* repeat-length (text on right-hand side of coastline represents low altitude; left-hand text represents high altitude).

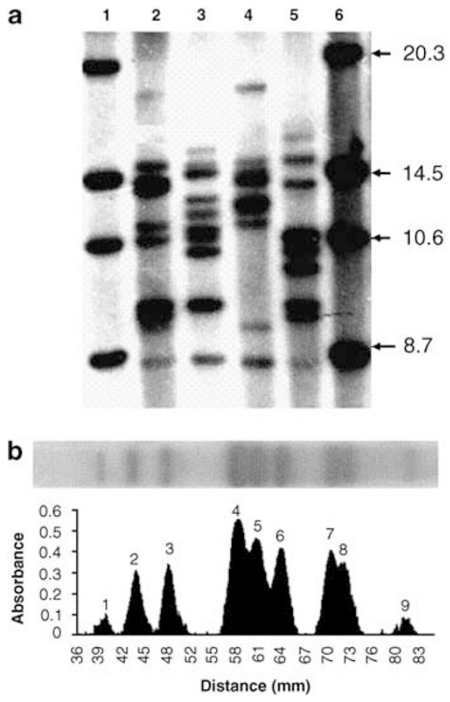

Figure 3 (**a**) Southern blot gel image of restriction-digested DNA probed with *hsr-omega* repeat sequence. Lanes 1 and 6 are standard size markers whereas lanes 2–5 show typical repeat-length variation each from five pooled isofemale lines. (**b**) Absorbance scan of lane 5 from (**a**) used to determine band positions and densities to weight allele groups.

were estimated by comparing the autoradiograph with marked origin to a captured image of the original gel, adjacent to a ruler scale, on which molecular weight size markers (Expand DNA mw marker XV; Roche Diagnostics, Castle Hill, New South Wales, Australia) were labelled with ethidium-bromide. The size standard allowed the placing of alleles into five groups: A ($>$20.3 kb), B ($>$14.5 to $\leqslant$20.3 kb), C ($>$10.6 to $\leqslant$14.5 kb), D ($>$8.7 to $\leqslant$10.6 kb) and E ($\leqslant$8.7 kb). The mean band size for each group being estimated at 20.8, 17, 12.6, 9.7 and 6 kb, respectively. These size means were used to estimate the average number of repeats for each group, corresponding to 63, 49, 34, 23 and 20 repeats, respectively (as each repeat is 283 bp in length and each fragment contains $\sim$3 kb of flanking region; Hogan *et al*., 1995).

To estimate the average number of repeats for a sample the allele group frequency (the proportion of bands falling in that group) was multiplied by the average repeat number for that group and these were summed across groups. Average repeat number for a location was the mean value of all isofemale lines. In 2001 and 2002 alleles were also grouped. However, to improve resolution total group band intensity was measured (the sum of the intensities of bands within each group–group intensities summing to one for the gel lane). This was performed by scanning bands from the X-ray film using a Bio-Rad imaging densitometer (Model GS-700) and weighted using Molecular Analyst Version 1.4 software that provided relative differences in band intensities (Figure 3b). The frequency of the allele group for the sample was estimated by dividing group intensity by average repeat number for that group (as the probe detects individual repeats and band intensity will depend on the number of repeats), summing these and expressing each group as a proportion. Average repeat number of a sample was then estimated as for 2000. This method only provides an estimate of average repeat number and frequency of an allele group for a sample as two different-sized fragments (with different repeat number) within a group are not discerned. However, this has proved to be an accurate and reliable protocol, especially for the 2002 collection and for the linkage disequilibrium study, when agarose gels were run for longer to resolve a larger number of different alleles and 10 different groups were distinguished (average band size and group repeat number being adjusted accordingly).

Average number of repeats for a location was analysed as a general linear model fitting year, latitude and year by latitude, using SPSS for Windows (14). Altitudinal variation of average number of repeats was assessed by paired comparisons of a high- with a low-altitude location at each of five latitudes (Figure 2) using a three-way χ^2 analysis (Cochrans–Mantel–Haensel (CMH) test; Sokal and Rohlf, 1996; SPSS 11.5). The Australian Bureau of Meteorology web site (www.bom.gov.au) was used to obtain six climatic variables (average daily maximum temperature for the hottest month, average

daily minimum temperature for the coldest month, average annual number of days above 30 °C, average annual number of days below 2 °C, mean monthly rainfall for the wettest month and mean monthly rainfall for the driest month). These were examined for association (correlation and partial correlation analyses using SPSS 14) with the average repeat-number data across all collection sites (3 years combined).

Family lines trait associations

Using a set of single-pair-mating family lines derived by Weeks *et al*. (2002), variation in *hsr-omega* repeats was examined for associations with three traits, cold tolerance (chill-coma recovery time), heat resistance (knockdown time) and wing size, traits that were characterized for these families three generations earlier (Weeks *et al*., 2002). Southern blots were carried out on DNA extracted from 25 adults from each family line which was scored for both the frequency of *fr* (*few-repeat)* alleles (=combined frequency of band groups containing bands <10.6 kb) and the frequency of *mr* (*many-repeat*) alleles (=combined frequency of groups containing bands >14.5 kb), determined by comparison to bands of standard sizes run on every gel. Data were analysed using both Pearson's coefficient and Spearman's coefficient of rank correlation in SPSS (11.5).

Cold-tolerant selection lines

A set of three cold-tolerant populations and three unselected controls, previously derived by Anderson *et al*. (2005b), were assayed for *hsr-omega* repeat variation, and for tissue levels of *hsr-omega* transcripts. Briefly, these independent populations were selected every second generation for fast chill-coma recovery time starting with a mass-bred population formed by pooling 25 isofemale lines derived equally from five low latitude sites on the north-eastern Australian coast (from latitudes between 16°01′ and 23°50′). DNA was extracted for the six populations at generation 22, when chill-coma recovery time averaged ~65% that of the control populations (Anderson *et al*., 2005b) and *hsr-omega* repeats quantified as described above. Levels of *omega-n* and *omega-c* were determined by real-time reverse transcriptase (RT)-PCR. A total of 3 replicate RNA extracts were made from all lines, each using 30 adult female flies and TriSure reagent (Bioline, Alexandria, New South Wales, Australia). Reverse transcription was completed with 2 μg of total RNA primed with 200 ng random primers (Invitrogen, Mt Waverley, Victoria, Australia) and using BioScript reverse transcriptase (Bioline). Real-time PCR was performed in the Rotor-Gene 6000 (Corbett Lifescience, Mortlake, New South Wales, Australia) for *omega-n* and *omega-c*. Measures were internally normalized to levels of *cyclin K* chosen because in microarray studies it showed particularly low variance across stress treatments, especially heat treatment, and it occurred at appropriate levels for *hsr-omega* transcript estimates (Sørensen *et al*., 2005). Duplicate PCR reactions using transcript-specific primers (400 nM) for *omega-n* (F—5′-TCCGCATTTATTTTTCTCCAC-3′, R—5′-GTGTATAGAATTTGGGACCTCCA-3′), *omega-c* (F–5′-TAGGAAGCCAGTGGGCGT-3′, R—5′-CCGAGTG CGTTTTCAGCA-3′) and for *cyck* (F—5′-GAGCATCCT TACACCTTTCTCCT-3′, R—5′-TAATCTCCGGCTCCCA CTG-3′) were completed for each cDNA batch using the Sensimix DNA kit (Quantace, Alexandria, New South Wales, Australia). Amplification conditions were 95 °C 10 min followed by 50 cycles of 95 °C (10 s), 58 °C (15 s) and 70 °C (5 s) with fluorescence data acquisition at the completion of the 70 °C step every cycle. Following cycling, product melt curves were generated by raising temperature from 69 to 90 °C in 1 °C increments. Primer pairs were previously validated across a range of cDNA concentrations with efficiencies found to be within the acceptable ranges. For transcript level analysis, Ct differences between *cyck* and *omega-n* and *cyck* and *omega-c* for each RNA extract were converted to fold differences and nested analysis of variance (ANOVA; SPSS 14.0) used to determine the effects of line and selection treatment.

Linkage disequilibrium

Adult *D. melanogaster* were collected from Coffs Harbour (30°19′S, 153°08′E) in March 2001 using banana bait traps and 50 isofemale lines (50) were set up at 18 °C using the field caught females. In order to score the haplotype of individual third chromosomes from this sample, a crossing scheme was established that allowed 78 independently derived chromosomes to be isolated. *hsr-Omega-L/S* scoring during the derivation of these lines allowed two independent chromosomes to be obtained from isofemale lines where the female was heterozygous. For each of the 50 lines several F1 males were single-pair mated to a virgin female carrying a chromosome three-balancer chromosome (TM6B). The parent F1 males were genotyped for *hsr-omega-L/S* to determine which lines to use for a second cross that would maximize the number of independent chromosomes assessed. Several male progeny that carried TM6B (with tubby and humeral phenotype) were individually crossed to virgin females from a stock that was homozygous for the markers of interest. This cross isolated independent chromosomes in separate fly lines. Within each line progeny that did not contain the balancer phenotype were used for scoring three markers on the right arm of chromosome three; *hsr-omega-L/S*, the closely linked microsatellite *DMU25686*, and *In(3R)P. hsr-Omega-L/S* and *DMU25686* were genotyped using previously described methods (Gockel *et al*., 2001; Weeks *et al*., 2002). *In(3R)P* was scored by polytene chromosome squashes and confirmed by a PCR-product gel assay (Anderson *et al*., 2005b).

Linkage disequilibrium between pairs of clinal markers was assessed using the standardized linkage disequilibrium coefficient *D*′ (Hedrick, 2000); *D*′ was calculated using the program Haploxt (Abecasis and Cookson, 2000) and the significance of linkage disequilibrium was determined using Fisher's exact test (Raymond and Rousset, 1995). *P*-values were corrected for multiple comparisons using the Sequential Bonferroni method (Rice, 1989). Linkage disequilibrium (*D*′) was also estimated between *hsr-omega-L/S* and *hsr-omega-repeats* in the family lines and significance assessed using Spearman's coefficient of rank correlation in SPSS.

hsr-Omega expression and cold shock

Three mass-reared laboratory populations were established 2 years prior to sampling, and kept in the laboratory at 25 °C in triplicate bottle culture for each

population. Each population was derived from an independent sample of ~50 adults from a homogenous pooling of 25 isofemale lines collected from several low-latitude (<24°S) and low-altitude (<100 m) coastal sites from Queensland, Australia. Three groups of 2 to 6-day-old females (~30) from each of the three populations were exposed to 0 °C for 6 h and allowed to recover for 20 min at 25 °C before being frozen for RNA extraction. For controls, three groups of un-exposed females from each population were collected in parallel. Real-time RT-PCR was used to determine levels of both transcripts, as described. To determine affects of population and cold treatment, nested ANOVA was performed for both *omega-n* and *omega-c*, separately (SPSS 14.0).

Results

Geographical variation

Within Australian *D. melanogaster* a large number of allele sizes were detected (Figure 3). It was not unusual to detect more than 10 different sized fragments, from a single population sample. The fragments from natural populations along the eastern coast of Australia varied from ~8 kb to at least 20 kb (~18 to ~61 repeat units, given that the fragments contained ~3 kb of flanking sequence) with an average of ~12 kb (~32 repeats).

The average number of repeats showed a clear linear effect of latitude ($F_{1,50} = 32.6$, $P < 0.001$) that was consistent across years (no year by latitude interaction, $F_{2,50} = 0.09$, $P = 0.91$), and did not depend on the year sampled (there was no effect of year, $F_{2,50} = 0.52$, $P = 0.60$). Quadratic terms did not improve the fit of the data. The association with latitude was negative; alleles with a lower number of repeats occurred more frequently at higher latitudes (Figure 4). We did not detect an effect of altitude on average number of repeats ($\chi^2_{CMH} = 0.77$, 1 degrees of freedom, d.f., $P > 0.05$), and no interaction with latitude occurred in a three-way χ^2 analysis. Using meteorological data assembled from all low-latitude sites (within 20 km of the collection sites) we looked for associations of average repeat number with four average temperature and two rainfall statistics. Although significant Pearson correlations occurred for all climatic variables, except mean monthly rainfall for the driest month, in partial correlation analyses these all became low and non-significant when latitude was controlled. Adding the high-altitude sites to these analyses did not significantly increase the levels of association detected.

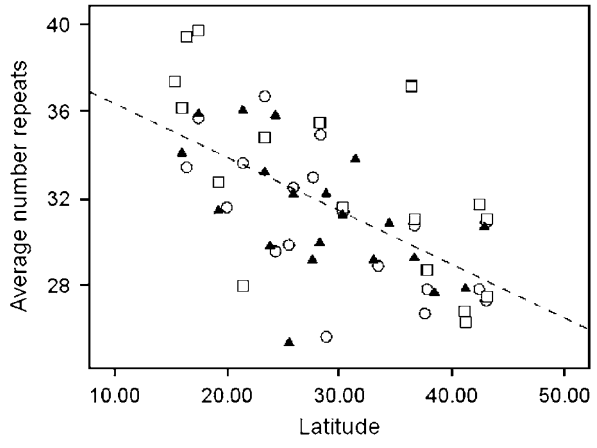

Figure 4 Association of average number of repeats with latitude in 2000 (○; $R^2 = 0.42$, $t = -3.48$, $P < 0.01$), 2001 (▲; $R^2 = 0.48$, $t = -3.72$, $P < 0.01$) and 2002 (□; $R^2 = 0.32$, $t = -2.91$, $P = 0.01$).

Family lines trait association

To look for any association of the repeat variation with thermal tolerance traits we characterized a set of family lines for *hsr-omega* repeat-number variation using Southern blots and measured chill-coma recovery time and heat resistance (Table 2). Families with a higher frequency of *few-repeat* (*fr*) alleles were more cold tolerant—their chill-coma recovery time was shorter (Figure 5), but there was no association of *fr* allele frequency with heat resistance. For the *many-repeat* (*mr*) allele group there was no indication of an association with either chill-coma recovery time or heat resistance.

We also examined *hsr-omega* repeat-number variation for association with wing area in the family line study as the earlier report on these lines (Weeks *et al*., 2002) found *hsr-omega-S* variation to be negatively associated with wing area (Table 2), and a major genetic factor that contributes to wing size variation maps to the vicinity of

Table 2 *hsr-Omega* genotype associations (Pearson's *r*) with three clinal traits for Coffs Harbour family lines

Trait	*Average number of repeats; hsr-omega-fr allele group frequency*	*hsr-Omega-S*[a]
Heat knockdown resistance time	0.020 (101); −0.057[b]	−0.085 (104)
Chill-coma recovery time	0.178* (102); −0.224[b,*]	−0.037 (104)
Wing area	0.193* (94); 0.234[b,*]	−0.472*** (96)

Numbers in parenthesis indicate number of family lines compared.
[a]Data from Weeks *et al*. (2002).
[b]Spearman's rank correlation coefficients.
*$P < 0.05$, ***$P < 0.001$.

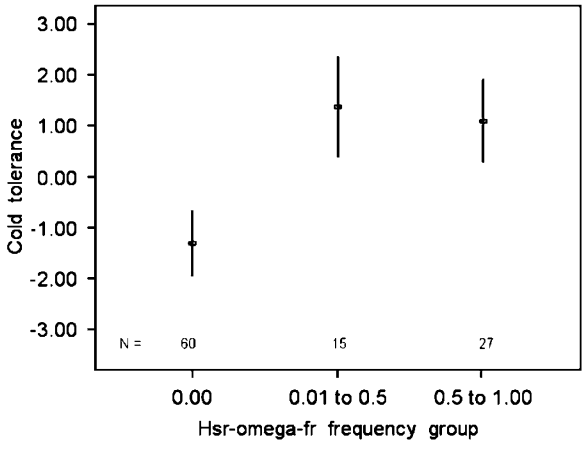

Figure 5 Associations in the Coffs Harbour family lines between *hsr-omega* repeat-number variation (lines placed into three *hsr-omega-fr* allele frequency groups) and cold tolerance (chill-coma recovery time (standardized) $x-1 \pm$ s.e.). The number of families per group are indicated (*N*). Analysis of variance (ANOVA) indicates a significant effect of *hsr-omega-fr* frequency group ($F_{2,99} = 3.32$, $P < 0.05$)—family lines with higher frequencies of *few-repeat* alleles having high cold tolerance.

Table 3 Number of *hsr-omega* tandem-repeat bands, the average number of repeats and frequency of markers *hsr-omega-L*, *In(3R)P* and *DMU25686* present in control (C1–C3) and cold-selected (S1–S3) lines

Line	*Average number repeats (s.e.)*	*Number of gel bands (= number of alleles)*	*Frequency*[a] *hsr-omega-L*	*Frequency*[a] *In(3R)P*	*Frequency*[a] *DMU25686 (most common allele)*
C1	40.44 (4.82)	5	0.84	0.15	0.15
C2	41.44 (11.39)	7	0.89	0.15	0.36
C3	44.07 (5.05)	6	0.65	0.33	0.34
S1	38.83 (3.16)	4	0.5	0.48	0.53
S2	38.73 (3.58)	4	0.79	0.24	0.5
S3	39.31 (4.85)	4	0.7	0.22	0.28

[a]$n = 30$ flies/marker/line.

hsr-omega (Calboli *et al*., 2003). Larger body size (often approximated as wing size) is recognized as an adaptation to cold-temperature environments and clinal variation in body size (Avis *et al*., 1997) has been associated with clinal variation in *In(3R)P* (Rako *et al*., 2006) that is in linkage disequilibrium with *hsr-omega-L/S* (Anderson *et al*., 2005a). A positive *hsr-omega-fr* allele frequency association with wing area was evident (Table 2). However, this was not robust as a partial correlation between *fr* allele frequency and wing size after controlling for *hsr-omega-S* was not significant ($r = 0.104$, d.f. $= 88$, $P = 0.16$). The partial correlation between *hsr-omega-S* and wing area, controlling for *hsr-omega-fr* allele frequency, remained highly significant, $r = -0.42$, $P < 0.001$ (d.f. $= 88$). For the *hsr-omega-mr* allele group there was no indication of an association with wing area. The data therefore suggest that *hsr-omega* repeat variation is independent of wing area.

Selected cold-tolerant populations and *hsr-omega* variation

A set of six populations, three selected for fast chill-coma recovery time and three non-selected control populations, were examined for *hsr-omega* repeat-number variation. Lines selected for cold tolerance showed a significantly lower average number of repeats when compared to unselected control lines (Table 3; $F_{1,4} = 8.96$, $P = 0.04$). There was also a significant line effect in these samples ($F_{4,30} = 3.81$, $P = 0.013$). No association of linked variation in *hsr-omega-L/S*, *In(3R)P* or the microsatellite marker *DM25686* occurred with the cold-selected or control populations (Table 3).

A significant difference in constitutive levels of the *omega-n* transcript between cold-selected and control lines (Figure 6a) was revealed by nested ANOVA ($F_{1,4} = 6.00$, $P = 0.031$), with no effect of line within selection treatments ($F_{1,12} = 0.76$, $P = 0.57$). On average, cold selected lines displayed a 14% reduction in expression compared to control lines. No differences in expression between lines or selection treatments ($F_{1,4} = 0.554$, $P = 0.498$) was detected for *omega-c*. Thus, two changes that reduce the number of *hsr-omega-n* transcript-bearing repeat units in tissues have occurred in the cold-tolerant populations due selection for cold tolerance—alleles with fewer repeat units have increased in frequency and the tissue level of this transcript is lower.

Linkage disequilibrium

In an attempt to tease apart associations between *hsr-omega* repeat variation, thermal traits and closely linked markers that also associate with these traits, linkage disequilibrium (D') was evaluated in a population recently collected from a location that is central along the latitudinal transect, Coffs Harbour. A central population was chosen because the markers also show latitudinal frequency clines and the rarity of genotypes present at high and low latitudes render significant disequilibrium difficult to detect. Fisher's exact test was used to determine the significance of pairwise comparisons between four markers that are closely

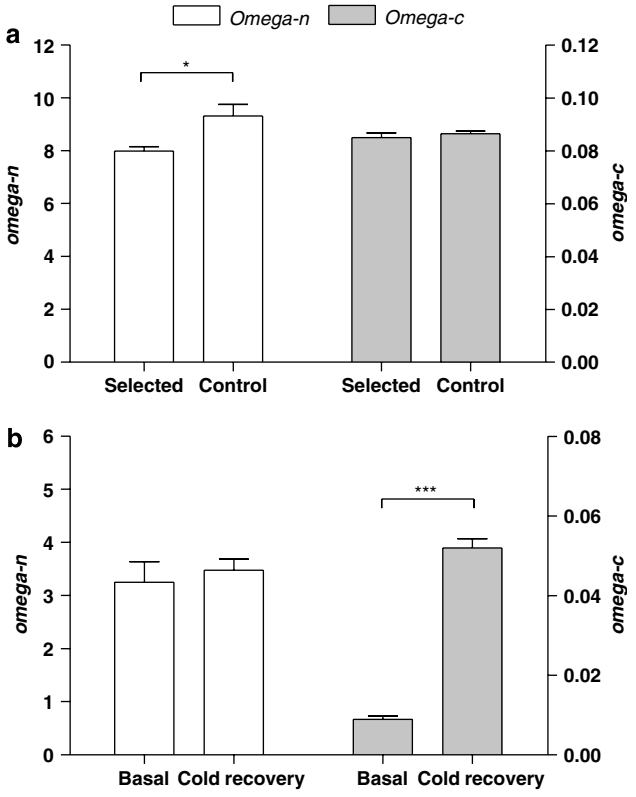

Figure 6 Cold treatment-related changes in levels of *omega-n* and *omega-c* transcripts determined by quantitative reverse transcriptase (qRT)-PCR. Bars represent the mean transcript levels normalized to *cyck* ± 1 standard error. (**a**) Comparison of the constitutive levels of both transcripts in adult tissues in cold-resistant (selected) populations vs cold-sensitive (control) populations. Only *hsr-omega-n* levels differed between selected and control populations ($F_{1,4} = 6.00$, $^*P = 0.031$). (**b**) Comparison of basal levels (adults held at 25 °C, no cold treatment) to cold recovery levels of both transcripts in adult tissues following exposure to 0 °C for 6 h, then recovery for 20 min at 25 °C for the set of three mass-reared laboratory populations ($F_{1,4} = 539.3$, $^{***}P < 0.001$).

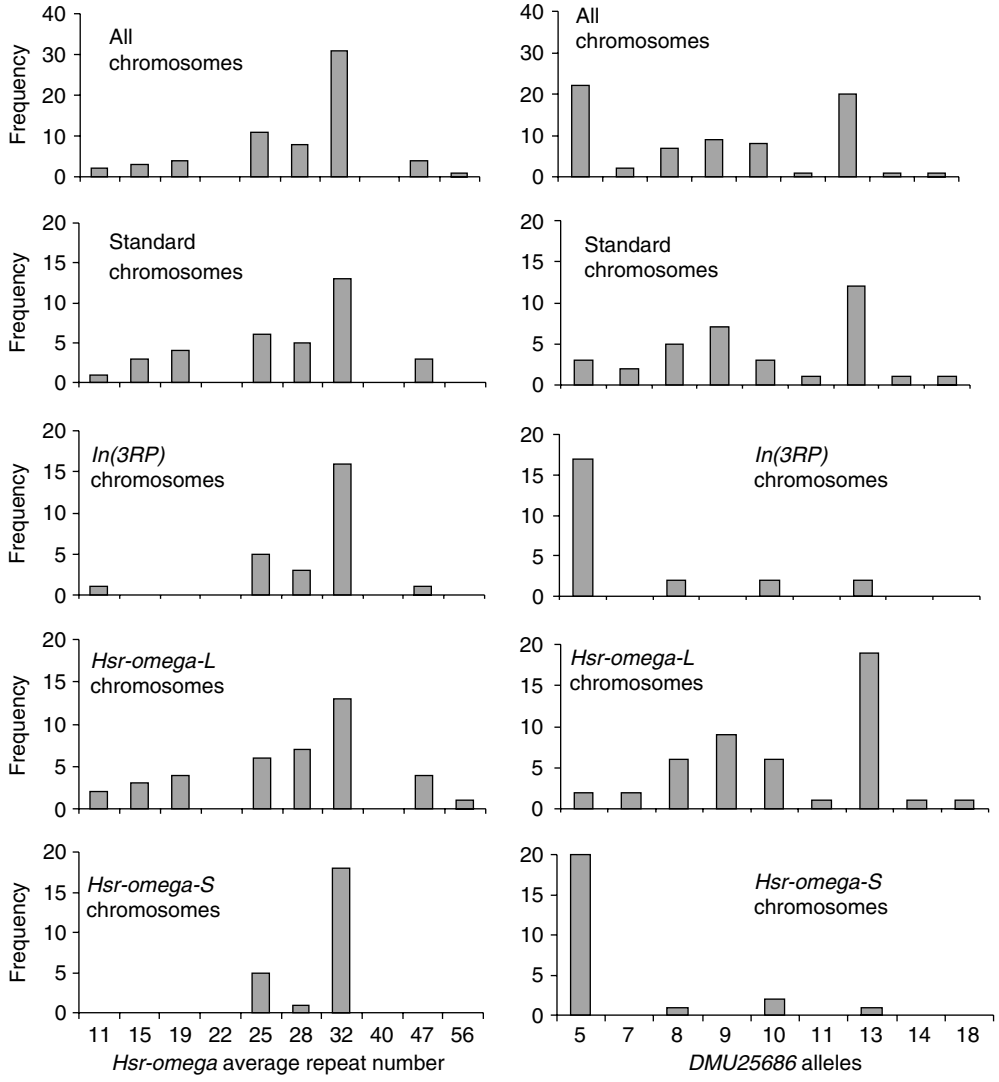

Figure 7 Gamete frequency distributions of *hsr-omega* repeat allele groups (on left-hand side, LHS), and *DMU25686* alleles (on right-hand side, RHS), on selected third chromosomes from the Coffs Harbour population.

linked on the right arm of chromosome 3, *hsr-omega* repeats, *In(3R)P*, *hsr-omega-L/S* and the microsatellite *DMU25686*. A significant linkage disequilibrium was observed between *hsr-omega-L/S* and *In(3R)P* ($D' = 0.72$, $P < 0.001$), as reported previously (Anderson *et al*., 2005a). Of 36 standard chromosomes sampled only 4 (11%) carried the *hsr-omega-S* allele, whereas 21 of 27 (78%) inversion chromosomes carried the *S* allele. Also, as depicted in Figure 7, strong linkage disequilibrium occurred between *hsr-omega-L/S* and *DMU25686* ($D' = 0.77$, $P < 0.001$), and between *DMU25686* and *In(3R)P* ($D' = 0.62$, $P < 0.01$). However, although *hsr-omega* repeat-number variation was not significantly associated with the inversion, or with *DMU25686*, it was mildly associated with *hsr-omega-L/S* ($D' = 0.57$, $P = 0.05$). There was a positive association between *hsr-omega-L* and allele groups that had both very low- and very high-repeat number (Figure 7). In this sample the *hsr-omega-S* chromosomes did not have repeat numbers in the high or low part of the range.

hsr-Omega expression and cold shock

To assess whether or not the transcripts of this gene were regulated in response to cold stress we quantified their levels in tissues 20 min into recovery from a 6-h exposure of adults to 0 °C, and compared them to that from adults held at 25 °C. Although no obvious change was detected in *omega-n* levels, a large increase occurred in the level of *omega-c* ($F_{1,4} = 539.28$, $P < 0.001$), about a sixfold increase (Figure 6b). Although our method of transcript quantification was not absolute the data suggest that the levels of *omega-n* were higher than that of *omega-c* (Figure 6), in contrast to a previous report where the levels detected were similar (McKechnie *et al*., 1998).

Discussion

To our knowledge repeat-number variation of the *Drosophila hsr-omega* gene from natural populations has not been previously investigated. Hogan *et al*. (1995) reported that the length of the repeat fragment that we

used in this investigation varied among laboratory stocks of *D. melanogaster*, from between 5 and 16 kb. Our data reveal that natural populations of this species, in Australia at least, contain large numbers of alleles with marked variation in repeat number, and with high levels of heterozygosity. Similar to the laboratory stock data of Hogan *et al*. (1995) the repeat fragment in Australian samples showed a lower length limit of ~5 kb, however, we observed a higher upper limit of about 20 kb of repeats. This is likely to be close to the upper limit for the species as over 300 isofemale lines were scored from a wide range of latitudes.

Our sampling has established that repeat-number variation shows a robust latitudinal cline along the eastern coast of Australia, with the average number of repeats being negatively associated with latitude. Temporal analysis of this cline shows that it is consistent over 3 years suggesting that average repeat number is relatively stable in Australian populations. As several other closely linked and highly variable genetic markers do not show latitudinal clines along this same Australian transect (Gockel *et al*., 2001) the stable latitudinal repeat cline suggests that natural selection is acting on this region of the chromosome. Note however that we did not detect any association between average repeat number and altitude, which one might have expected if environmental temperature was a factor that influenced the population frequency of different repeat-number alleles. However with altitude, as distinct from latitude, gene flow might account for a lack of differentiation over the shorter distances associated with habitat temperature variation. Also, we failed to detect any robust association of the *hsr-omega* repeat variation with any of the climatic variables assessed over the sampled distribution.

A cline itself does not exclude the possibility that *hsr-omega* repeat variation is adaptively neutral and a product of hitch-hiking with closely linked adaptive variation in another gene. The strongest candidates for such an adaptive marker, in linkage disequilibrium with the repeat variation, are the breakpoints of the cosmopolitan inversion *In(3R)P* (Kennington *et al*., 2006). However, we consider this is an unlikely possibility as we have shown that the repeat variation is largely independent of *In(3R)P*, despite being contained within it (Figure 7). Also, the repeat-number variation was not in linkage disequilibrium with a closely linked and clinally varying microsatellite marker *DMU25686*, that itself was in strong linkage disequilibrium with both *hsr-omega-L/S* and *In(3R)P*, consistent with a previous report (Weeks *et al*., 2002). Mild linkage disequilibrium was detected between the repeats and *hsr-omega-L/S*, however as depicted in Figure 7, this association seems 'non-directional' in that alleles that that have both few repeats and many repeats were both positively associated with the *hsr-omega-L* allele that occurs at high frequency only at higher latitudes (the *hsr-omega-S* chromosomes had an absence of both few and many repeats). Thus, if the *hsr-omega-L* allele is held at high frequency at cooler latitudes by selection this would not obviously result in high frequency of alleles with few repeats, assuming the same central-population pattern of linkage disequilibrium among these markers occurs at higher latitudes. Hence, our data are consistent with the idea that the *hsr-omega* repeat marker variation is largely independent of variation in the closely linked markers *DMU25686* and *In(3R)P*, and that a level of repeat disequilibrium with the clinally varying *hsr-omega-L/S* variation cannot 'explain' the cline in the repeats.

Although several independent data sets have shown an association of *hsr-omega-L/S* variation with heat knockdown tolerance (McColl *et al*., 1996; McKechnie *et al*., 1998; Anderson *et al*., 2003; Rako *et al*., 2007), and one QTL study (Norry *et al*., 2004) was consistent with an *hsr-omega* effect, some studies have not found *hsr-omega* variation to be associated with heat tolerance or heat stress. This may be because different measures of heat tolerance have been used (for example Morgan and Mackay, 2006) or because relevant microarray studies have used arrays on which *hsr-omega* is not represented (Leemans *et al*., 2000; Sørensen *et al*., 2005). Our central population family study gave no indication of an association of *hsr-omega* repeat variation with knockdown heat resistance. Although this might be because we only measured basal heat knockdown tolerance, not heat-hardened or mortality-based heat tolerance, these data did suggest an association between cold-tolerance and *hsr-omega* repeat variation—family lines that contained more *few-repeat* alleles had shorter chill-coma recovery times. This association is in the expected 'adaptive' direction as cold tolerance increases with latitude (Hoffmann *et al*., 2002), as does the frequency of alleles with low repeat number. Limited repeat variation data from a previous report is consistent with fewer repeats being associated with increased cold tolerance (measured both by reduced cold mortality and faster chill-coma recovery time; Anderson *et al*., 2003). However, we did not detect a strong association and further investigation was called for.

We therefore examined repeat variation in a set of three replicate lines selected for fast recovery from chill-coma for 22 generations, and in three non-selected control lines, and found an association in the expected direction, the cold-resistant lines had, on average, alleles with fewer repeats than the control cold-sensitive lines. Genotype variation between these cold-resistant lines and their controls indicated no association with the closely linked clinal markers, *In(3R)P*, *DMU25686* or *hsr-omega-L/S*. If the repeat variation and cold tolerance are causally related these findings for closely coupled markers are not surprising as linkage disequilibrium patterns are consistent with this interpretation. Also, the 'non-directional' linkage disequilibrium of the repeat and *hsr-omega-L/S* variations might explain why, when repeat association was detected between both cold-tolerance and *hsr-omega-L/S* variation, no association was detected between cold-tolerance and *hsr-omega-L/S* variation, both in these cold-selected populations and in the family line data.

The *hsr-omega* gene is clearly one of the *Drosophila* heat-stress genes as, like the heat-shock protein genes, it is upregulated in response to mild heat shock (Pardue *et al*., 1990). We asked if its regulation is also affected by cold treatment. Unlike other heat-shock puffs of *D. melanogaster*, the *hsr-omega* locus (at cytological location 93D) responds by puffing when a brief cold shock is given to warm-reared larvae (Singh and Lakhotia, 1984), and when cold-reared larval salivary glands are given a brief 24 °C warm-shock puffing occurs at the 93D locus and not at the puff sites producing heat-shock proteins (Lakhotia and Singh, 1985). However, puffing itself does

not definitively implicate the *hsr-omega* gene as several genes locate to the puff sites.

In this study we have shown upregulation of the *omega-c* transcript of *hsr-omega* following a 6-h exposure to 0 °C which suggests that the gene itself is involved in an adaptive response to cold. Although we only examined one time point, 20 min into recovery from the cold exposure, our data indicated a large increase in *omega-c* levels, and that this did not occur for the *omega-n* transcript. A temporal expression study during cold recovery in this species, as was recently performed by microarrays on which *hsr-omega* was not represented (Qin *et al*., 2005), is needed for both *hsr-omega* transcripts. We also found that constitutive levels of *omega-n* were reduced in replicate populations selected for cold tolerance, suggesting a role for *omega-n* in determining fitness following cold exposure. Both transcripts of this gene may help implement altered cellular processes that facilitate cold acclimation or efficient cold recovery, or both. Although our expression data implies a role for *hsr-omega* in a response to cold exposure, we cannot exclude the possibility that an unidentified closely linked gene in linkage disequilibrium with *hsr-omega* repeat variation has caused the association between repeat-number variation and cold tolerance.

Nonetheless to our knowledge this is the first study to demonstrate an association between genetic variation in *hsr-omega* repeats and both geographic and thermal environmental factors. Our data suggest that the repeat variation affects fitness in cold environments, that it is selected upon by some aspect of naturally occurring cold exposure, and that the consequence is a latitudinal genetic cline in frequency of alleles that encode different numbers of repeats—fewer repeats being favoured at colder latitudes. Just what cellular or physiological mechanisms might be responsible for such an association or process awaits further research. We can speculate however, as considerable background knowledge at the molecular and cellular level is available. The *hsr-omega* gene produces two major RNA transcripts, in all tissues, but it does not produce a detectable protein product. Although overall sequence divergence is not high across the *Drosophila* genus, gene structure and several short regions of sequence are highly conserved (Pardue *et al*., 1990). One such highly conserved sequence, across at least 12 *Drosophila* species, is a nonamer motif (AUAGGUAGG), two of which are imbedded in each 280 bp monomer of the repeats (Bardsley L and McKechnie SW, unpublished). Evidence suggests that these motifs, that are part of the nuclear transcript, may associate with heterogeneous ribonuclear proteins (hnRNPs) and help coordinate the availability of hnRNPs for messenger RNA processing (Zu *et al*., 1998). In relation to the latitudinal cline, populations at cooler latitudes on average have fewer repeating nonamers (shorter *omega-n* transcripts) and therefore would have few binding sites to sequester hnRNPs with possible downstream affects on rates of messenger RNA processing and protein synthesis. Such effects might be expected to increase when thermal stress impinges on the individual and the gene is upregulated. However, further work is needed to investigate cellular processes affected and concomitant fitness effects.

Regardless of any mechanism the data presented here are consistent with a model where-by *hsr-omega* repeat number is a causal factor underlying the established Australian latitudinal cline in chill-coma recovery time in this species. These data also suggest that *hsr-omega*, although recognised for some time as being involved in the cellular heat-stress response and being associated with heat-tolerance variation, may also be important in the cell's response to cold stress and influence the organism's performance in colder environments.

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