

Transcripts from the *Drosophila* heat-shock gene *hsr-omega* influence rates of protein synthesis but hardly affect resistance to heat knockdown

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Abstract While hundreds of genes have recently been implicated in an organism's response to thermal stress, our insight into the cellular and physiological mechanisms affected by these genes has advanced to a lesser extent. We focus on an enigmatic *Drosophila* heat stress RNA gene, *hsr-omega*, which encodes two RNA transcripts that are constitutively expressed in almost all developing and adult tissues, *omega-n* in the nucleus and *omega-c* in the cytoplasm; both being readily induced to high levels by mild heat stress. We derived three *hsr-omega* mutant lines via imprecise P-element excision and characterised them for changes in expression, in both the presence and absence of heat stress. Viability estimates indicate that a low level of *omega-n* is required for normal development. Consistent with the model of *omega-n* as a negative regulator of intron-processed mRNA levels the mutants displayed a 1.5-fold increase in rates of protein synthesis measured in ovarian tissue in the absence of heat stress, a result suggesting that an important function of *hsr-omega* is the modulation of general protein synthesis. The mutants had little effect on two measures commonly used to assess heat tolerance, heat-knockdown time and heat hardening ability, suggesting that more subtle heat-related fitness components need to be examined for effects of these mutations.

Keywords *Hsr-omega* · P-element excision · Protein synthesis · Heat tolerance · *Drosophila*

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Introduction

The genetic and cellular mechanisms that underlie heat-stress tolerance have recently been the focus of considerable research in diverse organisms (Larkindale et al. 2005; Collier et al. 2008; Healy et al. 2010), as has been the mechanisms that underlie heat acclimation (Horowitz 2001; Bernabucci et al. 2010). In this era of global warming these mechanisms are likely to be subject to adaptive evolutionary change (Deutsch et al. 2008; Hoffmann 2010; Thomas et al. 2004), and from conservational and agricultural perspectives, we need to understand the processes involved. *Drosophila* provides a useful model organism to study such mechanisms and processes (Lindquist 1980; Sgrò et al. 2010) and recent technological advances have revealed hundreds of new *Drosophila* genes that are likely to be involved (Sørensen et al. 2005; Laayouni et al. 2007). However, these genetic data have not yet led to equivalent advances in elucidating the cellular mechanisms affected by these genes. One enigmatic heat-inducible gene that is likely to affect *Drosophila* heat tolerance and heat-acclimation ability is *hsr-omega* (Pardue et al. 1990; McKechnie et al. 1998), despite the fact that just how this gene protects the cells has remained elusive (Lakhotia et al. 1999; Weeks et al. 2002). To address this issue we derive some new mutations in *hsr-omega* using imprecise P-element excision. We characterise the mutant strains for changes in gene transcript level, with and without heat stress, we test a current hypothesis about the cellular function of *hsr-omega*, and we look for effects of the mutations on two common measures of *Drosophila* heat tolerance.

While no protein product is known for *hsr-omega* its two RNA transcripts are constitutively expressed in nearly all tissues, *omega-n* in the nucleus and *omega-c* in the cytoplasm, and both are rapidly and significantly

up-regulated in response to thermal and other stresses (Pardue et al. 1990; Lakhotia et al. 1999). Growing evidence indicates that natural variation in genotype and expression of *hsr-omega* influence heat tolerance (McCull et al. 1996; Anderson et al. 2003; Rako et al. 2007; Collinge et al. 2008). The gene is particularly interesting in the context of heat acclimation or heat hardening as divergent changes occurred in the resting level of its transcripts in heat resistant populations, depending on whether they were heat-hardened or not prior to laboratory selection for heat resistance (McKechnie et al. 1998). The roles of the *hsr-omega* transcripts, however, and how they influence whole organism fitness under heat stress, is poorly understood, especially when compared to our understanding of the function of the heat stress proteins and the heat shock transcription factor (HSF).

Indications that *hsr-omega* affects whole organism heat tolerance come from early study of genomic deficiencies that uncover the gene (Lakhotia 1987; Mohler and Pardue 1984). However the insight provided has been limited because the genetic lesions also removed adjacent chromosomal genes (Gerasimova et al. 1995) rendering the causes of trait changes ambiguous, or because low viability of the lines precluded measurements of ecologically relevant traits such as adult heat-knockdown time (Hoffmann et al. 2002). Nonetheless, these aberrations have been useful in suggesting an important role for *omega-n* during heat stress. *Omega-n* regulates availability of nuclear factors known primarily to be involved in pre-mRNA processing (Lakhotia et al. 1999; Prasanth et al. 2000). Thus, we might expect lines differing in the levels of the nuclear transcript to have altered levels of general protein synthesis, and recent evidence supports this idea (Johnson et al. 2009a).

Levels of cellular protein synthesis are profoundly changed following heat shock (Ashburner and Bonner 1979; Lindquist 1980). In the nucleus, repression of general gene transcription (Spradling et al. 1975) and arrest of nascent transcript processing occurs (Mayrand and Pederson 1983) and these changes are paralleled in the cytoplasm by programmed shutdown of general protein synthesis and the induction of the protective heat shock proteins (Yost and Lindquist 1986). Both the levels of heat shock proteins and the extent of shutdown of general protein synthesis following heat stress changed in lines successfully selected for increased or decreased heat tolerance, demonstrating a link between these processes and heat-stress adaptation (Stephanou et al. 1983). Further, levels of protein synthesis following heat stress have been associated with the capacity of a line to increase knockdown time when heat hardened (Johnson et al. 2009b). If *hsr-omega* is able to modulate levels of protein synthesis, it may influence variation in thermal tolerance via an effect on protein synthesis.

Here, we generate three new alleles of the *hsr-omega* gene by excision of a P-element insertion (*EP3128*) in close proximity to the *hsr-omega* transcription start site. Lines homozygous for these mutations have been characterised for *hsr-omega* expression with and without heat-induction, for rates of protein synthesis, for viability and for heat tolerance measured as adult knockdown time following two different levels of heat stress. We report that a markedly reduced level of both of the main *hsr-omega* transcripts is not necessarily lethal, but it disrupts development, affects rates of protein synthesis and has little effect on adult heat-knockdown time.

Materials and methods

Fly maintenance and lines used

Flies were maintained at 25°C under a 12 h light regime in 42 ml vials or 250 ml bottles containing potato-dextrose medium. Flies were raised under constant density for testing (Clancy and Kennington 2001). P-element insertion line *EP3128* (Rørth et al. 1998) was sourced from Szeged stock centre (Hungary). The transposase source used was *w¹¹¹⁸; Sb, P[ry⁺, Δ2–3]/TM6*.

Derivation of mutant lines

For P-element excision mutagenesis, line *EP3128* was selected due to the elements close proximity to the *hsr-omega* transcriptional start site. Firstly, the transposase source ($\Delta 2-3$) was crossed to *EP3128/TM6B en masse* and the progeny containing both the P-element and transposase chromosomes crossed again to the transposase source. Individual excision progeny were crossed back to the transposase source to generate balanced (TM6B) lines. Once 200 independent excision lines had been established, DNA was isolated using a standard phenol–chloroform method and each line was subject to PCR with primers designed to detect deletions not likely to affect the upstream gene (Fig. 1a, F-5' TGT AGG TCC TTG TTG CCA AAC, R-5' GGA GAG AGA GAA CGA GCC AGT). Amplicons smaller than that expected from wild-type (3.3 kb) were gel-extracted (GeneClean II, Qbiogene) and sequenced (BigDye v3.1 mix, Applied Biosystems 3730S Genetic analyser) to determine the nature of excision events. For precise excisions, additional sequence was obtained using an extra reverse primer (R-5' TCC GAG GGA CTA TTC TCG TAA) to survey the region for evidence of other local sequence aberrations. All sequences were analysed for the creation of a new transcription start site for *hsr-omega* using McPromoter for *Drosophila* (Ohler 2006). Imprecise excision lines (56, 66) and a

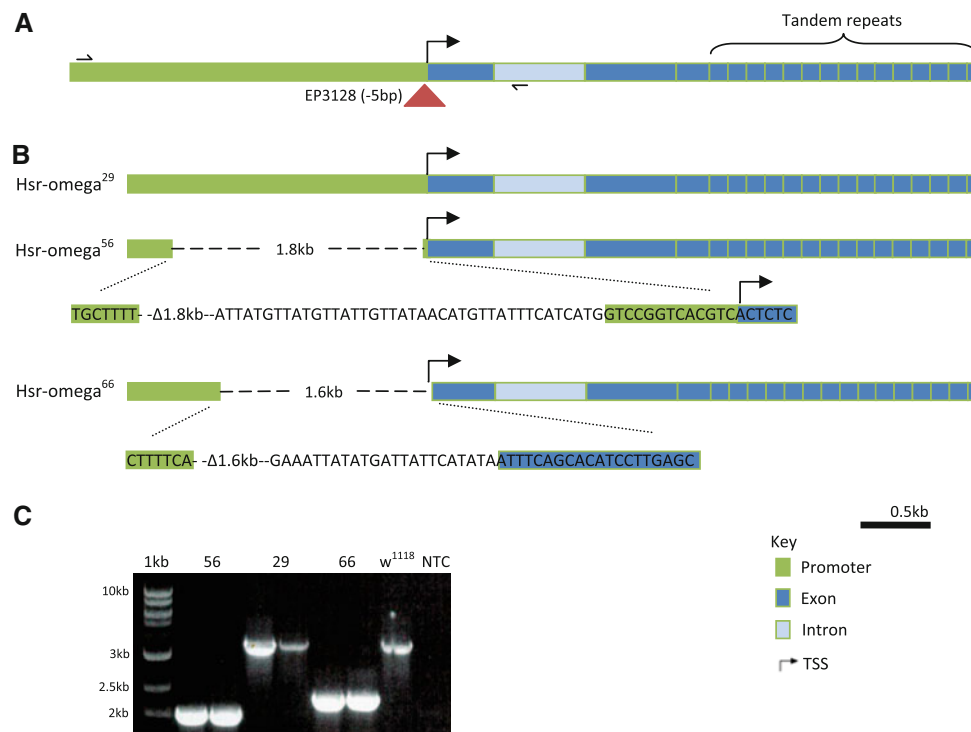


Fig. 1 The *hsr-omega* gene and nature of excision alleles derived via P-element mobilisation. Relative position of the P-element insertion in line *EP3128* (triangle) and structure of the *hsr-omega* gene including the 5' promoter region (left of arrow, green), exons (right of arrow, blue) and intron (right of arrow, pale-blue) are displayed (TSS transcription start site). Small arrows represent primer-binding sites used for PCR screen of P-element excision events (a). Alleles derived from a precise excision event (*hsr-omega*²⁹) and imprecise excisions

events *hsr-omega*⁵⁶ (Δ 1,853 bp) and *hsr-omega*⁶⁶ (Δ 1,598 bp) are detailed with their respective genomic lesions indicated by dashed lines (b). The sequences below (unhighlighted) are the sequences of fragments found to be inserted in place of deletions. Agarose gel (1%) separation of duplicate PCR products from excision line homozygotes using aforementioned primers (c). Wild-type expected product size is \sim 3.3 kb. DNA marker is 1 kb (Promega); NTC no template control

precise excision line (29) for use as a control, were maintained with a floating *TM6B* balancer chromosome. Homozygous mutant lines were derived at least two generations prior to use in all tests.

Quantification of *hsr-omega* transcript levels

Levels of *omega-n* and *omega-c* were estimated by real time RT-PCR as previously described (Collinge et al. 2008; Johnson et al. 2009a). Transcript specificity was assured by primer placement; for *omega-n* both primers were downstream of the full *omega-c* template (and upstream of the repeat region), and for *omega-c* the forward primer spanned the intron splice junction while the reverse primer was close-by (near the 5' end of the *omega-c* template, as depicted in Johnson et al. 2009a). Three replicate RNA extractions were made for each line for both basal and heat-shocked treatments. Replicates consisted of twenty adult females reared in separate culture vials and aged 5–7 days. The heat shock was performed by placing flies in capped

10 ml centrifuge tubes, before submerging in a circulating water-bath set to 36.0°C for 1 h. RNA was isolated from replicate groups using TriSure reagent (Bioline) and DNase treated (New England Biolabs) for 40 min at 37°C prior to cDNA conversion. For realtime RT-PCR, each run consisted of one replicate from each line for the two treatments with *omega-n* and *omega-c* run separately in addition to the reference gene, *Cyclin K*. Amplification conditions and primer sets for all genes were validated across a range of cDNA concentrations, and melt curves analysed prior to transcript quantification.

Viability

Homozygous adults from each line and line *w*¹¹¹⁸ were allowed to mate and lay overnight on culture media containing food dye for ease in embryo picking, and three times the standard agar amount to keep eggs above the media surface. Ten replicate groups of twenty eggs each were picked and carefully transferred to 42 ml culture vials

containing fresh media supplemented with ampicillin (50 mg/ml) to minimise infection. Vials were placed at 25°C and scored only for both the number individuals surviving to pupate and the number of adults to emerge.

Rates of protein synthesis

Protein synthesis rates were assessed by measuring the amount of ^{35}S -methionine incorporated into dissected ovarian tissue in 1 h, as previously described (Johnson et al. 2009a). For each line (w^{1118} and lines homozygous for 29, *EP1328* and 56) rearing occurred in parallel at 25°C at controlled densities in five separate culture vials in which adults were allowed to mate until 5–7 days old. For each line, between seven and nine replicates of four females (up to two replications per culture vial) were lightly anaesthetised under CO_2 , and their intact ovaries immediately dissected for pulse-labelling. Immediately following the 1 h labelling reaction samples were thoroughly washed and centrifuged (three times on ice) to remove unincorporated ^{35}S -methionine (Johnson et al. 2009a). Each block of replication included all lines and the experiment was complete within 12 h. Counts per minute (CPM) of radioactive emissions were corrected for block effects by multiplying each by grand mean/block mean. Incorporation of label into protein and its successful recovery were confirmed using autoradiography by the detection of labelled protein bands from the supernatant run on electrophoretic gels. In addition, a control assay in which ovaries were pre-incubated with 3 mM cycloheximide (a protein synthesis inhibitor; Sigma-Aldrich Co, St Louis, C-7698; Bendena et al. 1989; Marcos et al. 1982) at 25°C for 20 min prior to and during labelled methionine incorporation confirmed that >75% of recovered label was due to protein synthesis. To account for line differences in ovary mass, radiolabel incorporation was standardised against total ovary protein measured in a separate set of females at the same age and raised under identical culture and density conditions as those used for radio-labelling. Three replicates per line each consisting of ovaries from ten dissected females were added to 100 μl of phosphate buffered saline, supplemented with anti-protease agents (2 mM phenyl methyl sulphonyl fluoride, and 1% (v/v) anti-protease cocktail (100 $\mu\text{g}/\text{ml}$ pepstatin A, 50 $\mu\text{g}/\text{ml}$ leupeptin, 10 mM benzamidine, 10 mM sodium metabisulphite)) in a centrifuge tube. Each replicate was thoroughly homogenised then centrifuged. The protein content of each replicate was assessed using the bicinchoninic acid (BCA) protein assay (Pierce Biochemicals). Lines differed in total ovary protein mass ($F_{3,8} = 13.8$, $p < 0.02$), so rates of protein synthesis were expressed as counts per minute per microgram of ovarian protein.

Heat tolerance

Heat tolerance assays, using w^{1118} and homozygous derived lines, were conducted in a similar manner to Hoffmann et al. (2002) but at both a mild and a severe stress temperature (37 and 39°C, respectively) and both with and without a hardening pre-treatment. For each heat-stress assay, all lines were reared in parallel at controlled densities. Adults aged 5–7 days were used with sexes separated under light CO_2 at least 2 days prior to testing. For the hardening treatment, flies were exposed to 37°C for 1 h in 10 ml centrifuge tubes, then transferred to food vials and left to recover for 6 h before testing. Both untreated and hardened flies were tested within a single run in both assays and sexes run separately. For the 39°C assay, four flies of a single sex (either all hardened or not) were placed in a 33 ml sealed glass vial and submerged in a clear glass tank set to 39°C. Two runs for each sex were necessary to assay five replicate vials of hardened and untreated flies for all lines, with line, treatment and vial position randomised across all runs. The time taken for each fly to reach heat-induced paralysis was recorded. Similar randomisation of line, replicate and treatment occurred for the 37°C assay, except one fly was tested per vial and runs were repeated until approximately ten flies had been tested from each line and for each treatment. Sexes were run separately.

Statistical analyses

Effects of heat hardening, temperature and *hsr-omega* expression disruption on thermal tolerance were assessed using univariate one-way and two-way ANOVA functions in SPSS 17.0 for Windows. Sexes were analysed separately. Differences between lines for transcript levels were detected by Dunnett's multiple comparison t tests, and effects on viability and heat tolerance were assessed using unpaired t tests comparing mutant lines individually to the precise excision *line 29*.

Results

By mobilising the *EP3128* P-element three independent *hsr-omega* excision lines were selected from about 200 lines for further characterisation, one (*line 29*) with a precise excision and two (*lines 56* and *66*) that had sizeable genomic lesions as detected by the size of a PCR product generated from the proximal region of the promoter (Fig. 1c). Sequencing revealed that the deletion in *line 56* included 1,853 bp of upstream 5' regulatory sequences (from -13 bp), whereas the 1,598 bp deletion of upstream regulatory sequences in *line 66* removed 9 bp of transcribed sequence including the transcriptional start site

(TSS) (Fig. 1b). Sequence analysis indicated deletions of the TATA box (−31 bp), initiator sequence, and known heat-shock and GAGA elements in both lines (Mutsuddi and Lakhotia 1995; Ryseck et al. 1987). Additionally, short sequences of 41 and 24 bp were found inserted in place of the 56 and 66 deletions, respectively (Fig. 1b), likely resulting from the incorporation of excised fragments during DNA break repair. Promoter prediction analysis of the regions upstream of *hsr-omega* including these short insertions in the deletion lines suggested no new TSS was created in either excision event (data not shown). For the precise excision event, sequencing that extended from the gene located upstream until the beginning of the *hsr-omega* tandem repeat region confirmed the restoration of an intact promoter and 5' gene region.

Expression levels of *omega-n* and *omega-c*, with and without heat exposure, were markedly affected in

homozygous lines *EP3128*, *56* and *66* (Fig. 2). Precise excision of the P-element (*line 29*) restored near normal levels of both transcripts under basal and heat-shock conditions as assessed by comparison to *w¹¹¹⁸*. The EP element itself (8 kb) inserted 5 bp upstream from the *hsr-omega* TSS (*line EP3128*) reduced basal *omega-n* and *omega-c* to 10–15% of normal. Notably, *omega-c* in *EP3128* was still heat-inducible to roughly double that of basal levels ($t_4 = 3.19$, $p = 0.017$, see inset Fig. 2b), however, not close to the 16-fold induction observed for the control line *w¹¹¹⁸*. Heat-induction of *omega-n* was not significant for *EP3128* ($t_4 = 1.06$, $p = 0.17$). The deletion in *line 66* completely abolished expression of both transcripts as their amplification levels were not different from the background levels observed in no-template control reactions. While no *omega-c* was detected in *line 56* (see insets of Fig. 2b), low levels of *omega-n* were consistently observed

Fig. 2 Disruption and rescue of expression levels of the heat-inducible transcripts *omega-n* (a) and *omega-c* (b) determined by qRT-PCR. Both basal (*white*) and heat-induced (36°C 1 h, *grey*) transcript levels in adult females are shown. [^]Indicates not significantly different from zero levels as determined by comparison to negative control reactions (data not shown). Significant differences between *w¹¹¹⁸* (control) and other lines within each treatment (ANOVA, Dunnett's test, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$) are indicated above columns. Error bars represent +1 standard error, $n = 3$

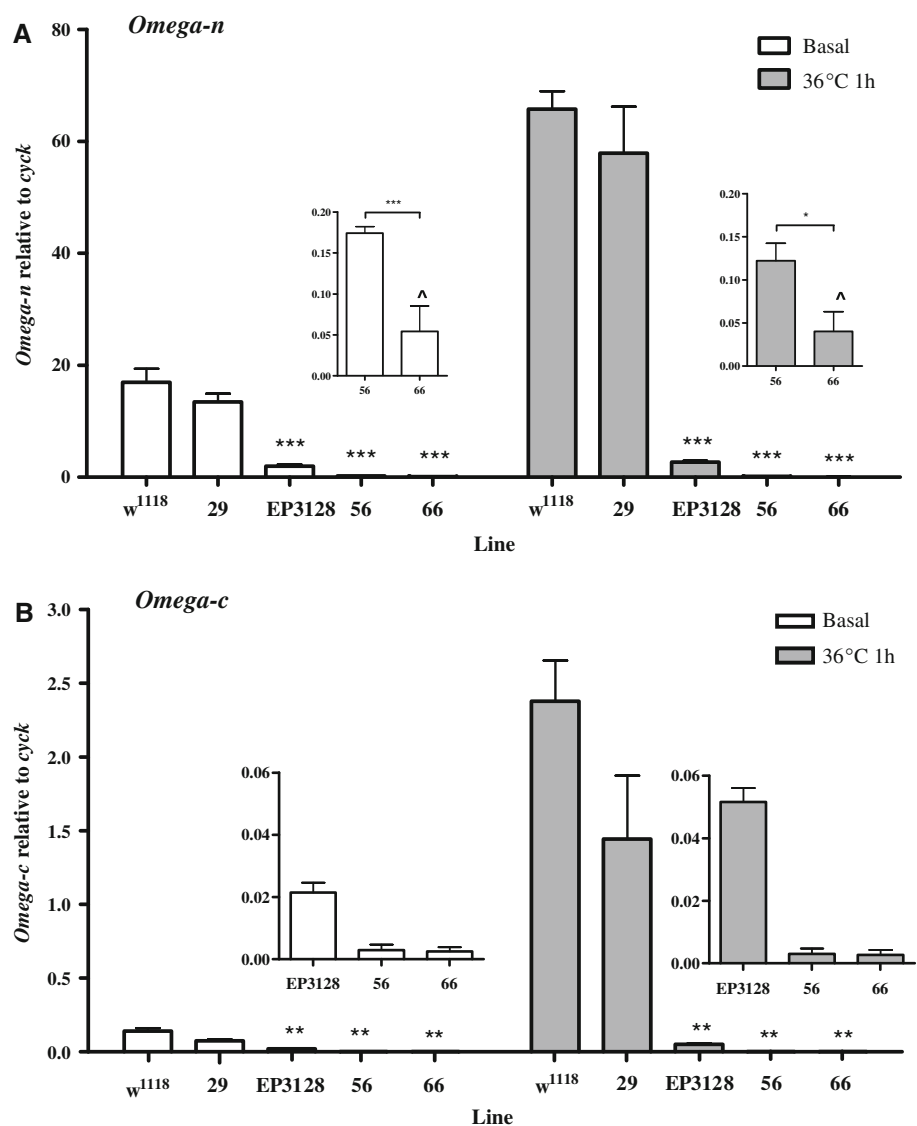
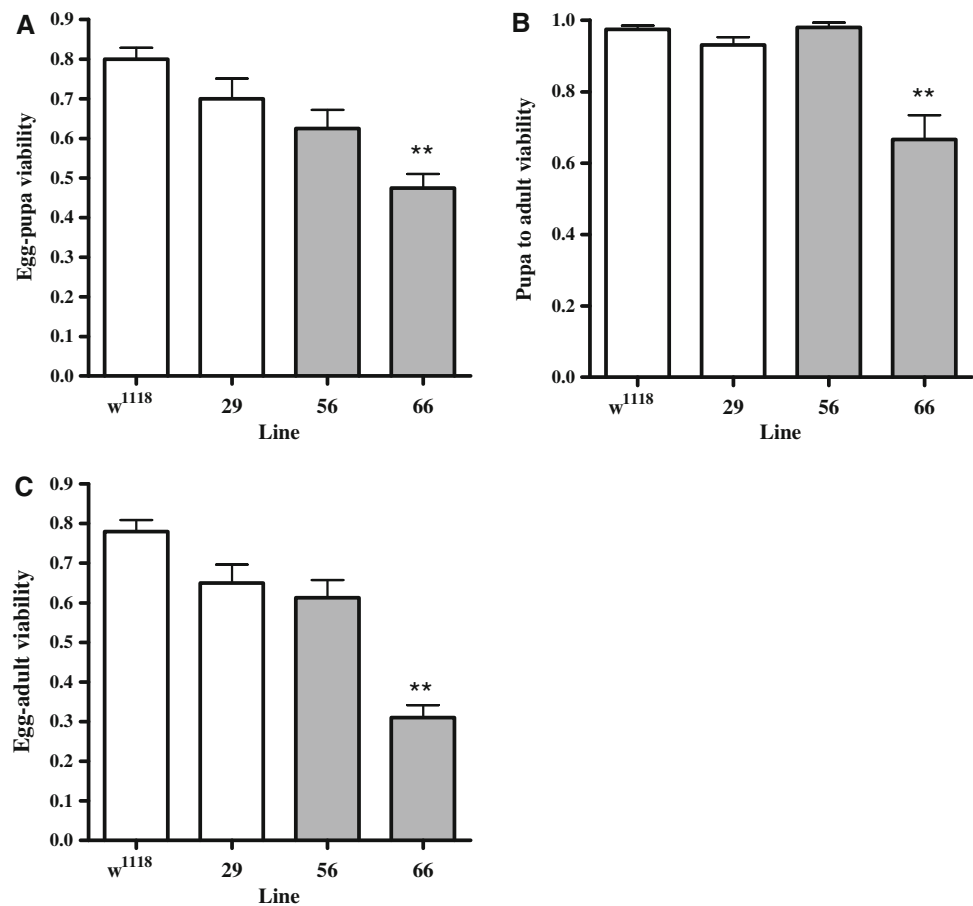


Fig. 3 The affect of genomic deletions at the *hsr-omega* locus on viability. Viability was assessed at 25°C from egg to pupa (a), pupa to adult (b) and egg to adult (c). Comparisons are made between excision lines (grey) and control line 29 (two-tailed *t* test, $**p < 0.01$, $***p < 0.001$). Error bars represent +1 standard error, $n > 8$ in all cases



(insets of Fig. 2a). *Omega-n* in line 56 compared to control line *w*¹¹¹⁸ had about 1% of basal levels and 0.1% of heat-shocked levels as no increase of *omega-n* following heat treatment was detected ($t_4 = 0.06$, $p = 0.478$).

Balanced cultures of line 66 (on *TM6B*) displayed noticeable levels of recessive lethality. Nonetheless, surviving homozygotes were fertile and able to be cultured. Viability tests confirmed semi-lethality in line 66 (Fig. 3) where almost half of line 66 individuals died at pre-pupal stages, and of those that survived to pupate, only 67% emerged as adults compared to 93% and 98% for lines 29 and *w*¹¹¹⁸, respectively. Line 66 was difficult to work with as very few healthy individuals could be obtained; adults were slow moving and cultures were susceptible to bacterial infection. In contrast, line 56 homozygotes showed no obvious affects of the deletion, as there was no difference in pupa-to-adult viability (Fig. 3). Cultures of line 56 were healthy and fertile. Examination of line 56 ovaries from several females confirmed all stages of oogenesis to be present and in ‘normal’ proportions (data not shown).

Protein synthesis rates were determined for two lines with reduced *hsr-omega* expression, homozygous lines *EP3128* and 56, and two control lines, *w*¹¹¹⁸ and homozygous line 29, and significant differences were detected (Fig. 4) as

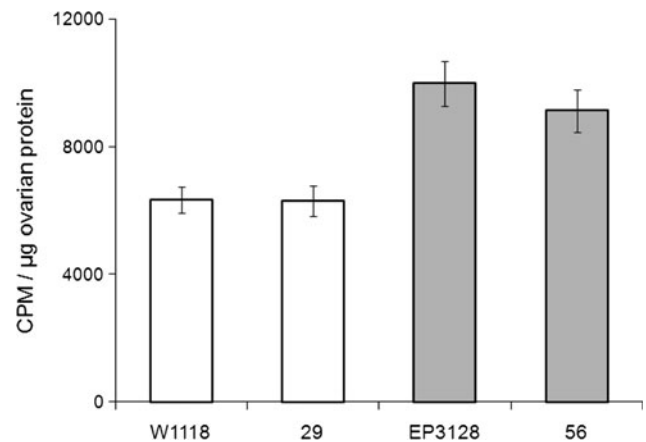


Fig. 4 Protein synthesis rates of control lines (*w*¹¹¹⁸ and 29) and lines with altered *hsr-omega* expression (*EP3128* and 56). Dissected ovaries were metabolically labelled with ³⁵S-methionine for 1 h. Rates are measured as counts per minute (CPM) per microgram ovarian protein. Error bars represent ± 1 standard error

assessed by one-way ANOVA ($F_{3,31} = 12.00$, $p < 0.001$). A Tukey post hoc test clearly identified two homogeneous subsets of two lines each, the control lines (*w*¹¹¹⁸, 29) and the mutant lines (*EP3128*, 56). Each line was significantly different to both lines in the alternate subgroup (all $p < 0.01$).

The marked down-regulation of *hsr-omega* expression in the mutated lines has increased the levels of protein synthesis in ovarian tissues by about 1.5-fold.

Effects of the homozygous *hsr-omega* mutations on heat-knockdown time were measured at 37°C and 39°C and analysed separately, as were the sexes. Note that average knockdown times were reduced from about 80 min when exposed to 37°C down to 20 min at 39°C. When tests were conducted at the milder temperature our hardening treatment did not significantly increase knockdown time, in both females (ANOVA, $F_{1,160} = 3.51$, $p = 0.063$) and males ($F_{1,136} = 0.006$, $p = 0.937$), so the treatment data were pooled to assess the effect of disrupting *hsr-omega*. Females from *line 56* had a slightly reduced tolerance to 37°C compared to control lines, and while *EP3128* also knocked down sooner, the result was not significant (Fig. 5a). In males, however, mutant lines were not significantly different from controls (Fig. 5b). In contrast, for the severe heat stress tests at 39°C, heat hardening caused a significant increase in knockdown time in both females and males (Fig. 6; females: $F_{1,36} = 13.86$, $p = 0.001$; males: $F_{1,36} = 23.55$, $p < 0.001$). For both unhardened and hardened treatments, however, the effects of the mutations on knockdown time were not evident. Consideration of the two *hsr-omega* disrupted lines only, *EP3128* and *line 56*, left no doubt that the heat hardening treatment of the 39°C test was effective at increasing knockdown time in the disrupted lines ($F_{1,16} = 14.70$, $p < 0.001$ for males and $F_{1,16} = 12.98$, $p < 0.01$ for females).

Discussion

A high rate of P-element transposition into the *hsr-omega* gene in screens following P-element mutagenesis has provided numerous lines that could potentially disrupt expression of *hsr-omega*. We worked with *line EP3128* that had potential to severely affect *hsr-omega* function due to the proximity of the insertion to the transcription start site (TSS). Homozygotes for *EP3128* were healthy and fertile, unlike those previously described to lack *hsr-omega* expression (Mohler and Pardue 1984), suggesting that *hsr-omega* was still functional. This proved to be the case as expression of both *omega-c* and *omega-n* in *EP3128* were not abolished, although they were markedly reduced. In addition, *omega-c*, but not *omega-n*, remained heat-inducible, suggesting the heat shock regulatory elements remain intact despite being shifted ~8 kb (the size of the P-element) upstream. The *hsr-omega* gene appears to have a striking capacity to remain functional even with large insertions. This is relevant given the high frequency of P transposable element insertions observed in the *hsr-omega* promoter in natural populations (Walser et al. 2006) and

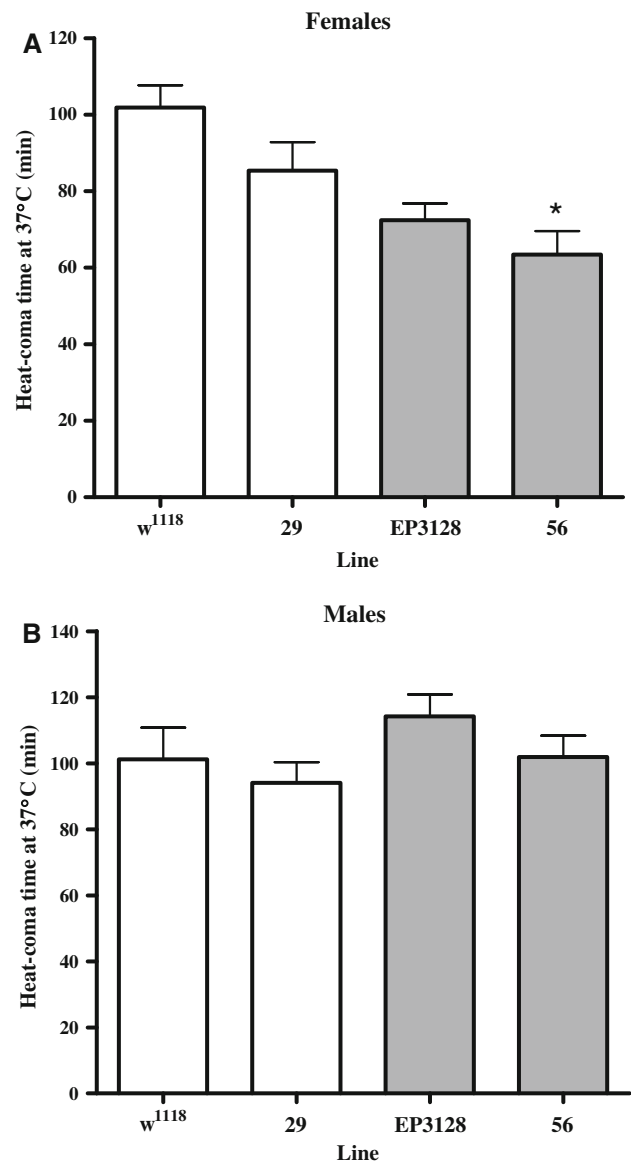
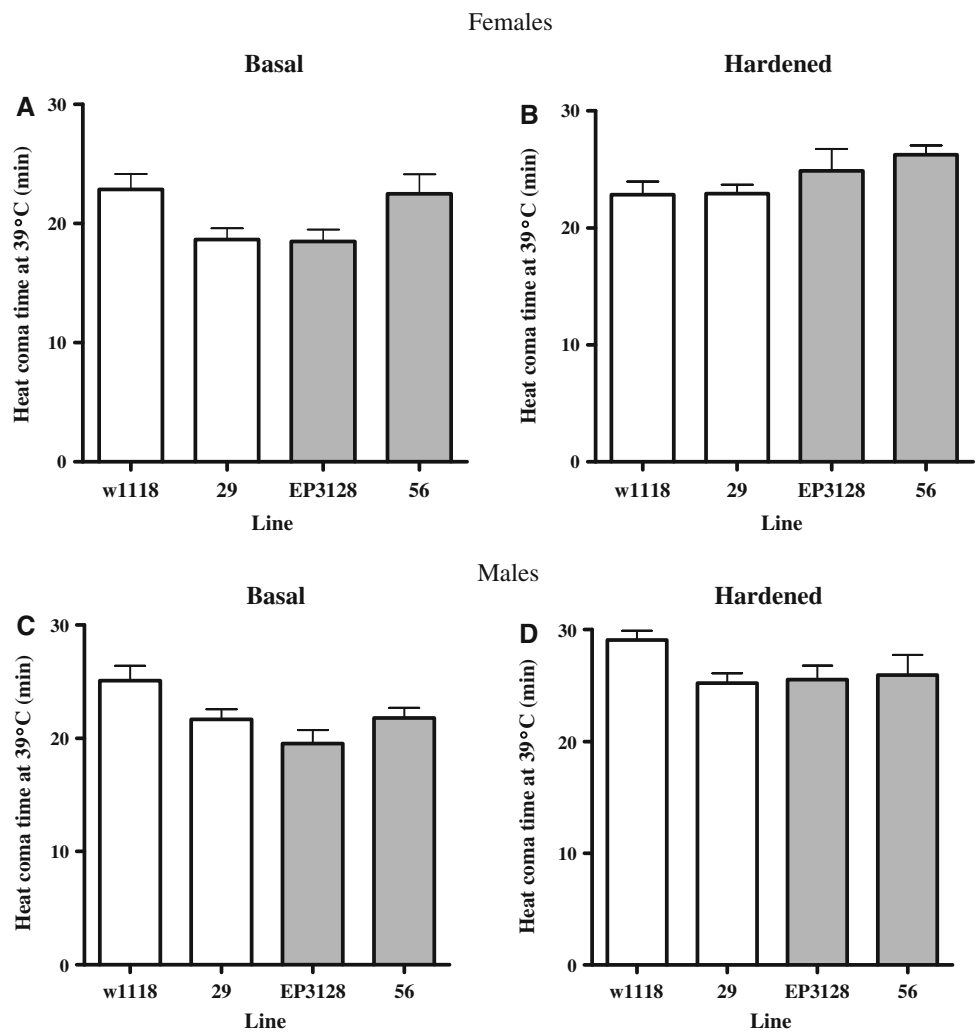


Fig. 5 Mild-temperature heat tolerances in *hsr-omega* mutant lines. Heat tolerance was measured by the heat-knockdown test at 37°C in females (**a**) and males (**b**). No affect of hardening treatment was detected for females (two-way ANOVA, $F_{1,160} = 3.51$, $p = 0.063$) or males ($F_{1,136} = 0.006$, $p = 0.937$), so treatment data were pooled to test genotype. A significant difference between control *line 29* and *hsr-omega* mutant *line 56* is denoted above column (one-tailed *t* test $*p < 0.05$). Error bars represent +1 standard error

suggests that these insertions could result in significant natural variation in *hsr-omega* expression levels. Fine control of *hsr-omega* transcript levels in *Drosophila* adults appears not to be important for ‘normal’ development and viability.

To increase the chance of recovering deletions that included both a substantial portion of the *hsr-omega* regulatory region and of transcribed sequence a large number of independent excision lines (>200) were derived from

Fig. 6 High-temperature heat tolerances in *hsr-omega* mutant lines. Heat tolerance was tested at 39°C in females (a, b) and males (c, d). Basal (a, c) and hardened (1 h at 37°C followed by 6 h recovery, b, d) heat tolerance is shown. No significant differences between control (line 29) and *hsr-omega* mutant lines (*EP3128* and line 56, grey) were detected. Error bars represent +1 standard error



EP3128. The deletions in *lines 56* and *66* that removed core and responsive *hsr-omega* 5' regulatory sequences (Garbe and Pardue 1986; Garbe et al. 1989) achieved this end. In *line 66*, the TSS was also deleted and no expression of *omega-n* nor *omega-c* was detected. However, *line 56* while having no detectable *omega-c* had low but detectable levels of *omega-n*. How does *line 56* achieve *omega-n* transcription given almost complete lack of promoter sequence? The presence of effective regulatory signals within the transcribed region of *hsr-omega* cannot be ruled out, although no consensus downstream promoter element (DPE) or motif ten element (MTE) appear to be present (Lim et al. 2004). In addition, it is possible that such a signal occurs within the short sequence found in place of the genomic deletion (Fig. 1b), however promoter prediction analysis suggests this is unlikely. The non-inducibility of *omega-n* in *line 56* following heat shock is consistent with the absence of heat-shock elements in this line (Pelham 1982).

Gross differences in fitness occurred between homozygous cultures of *lines 56*, that was healthy, and *66* that was not. Explanations other than *hsr-omega* expression differences, such as a variable genetic background or multiple P-element insertions in *EP3128*, are unlikely since all lines derived in this manner retain *w¹¹¹⁸* chromosomes and few EP lines have more than one insert (Berkeley Drosophila Genome Project). The development characteristics of these *hsr-omega* mutations are notably less severe than those described by Mohler and Pardue (1984) for their *e^{GP4}/GC14* deficiency trans-heterozygotes, and suggest that the additional lethality of their trans-heterozygotes is likely to be associated with other genes that were also disrupted by these chromosomal deficiencies. Our data indicate that only low concentrations of *omega-n* are enough for successful larval viability, pupation, and reproductive performance, as occurred in *line 56* that had no detectable *omega-c*. While complete loss of both transcripts, as occurred in *line 66*, was not necessarily lethal and did not

remove reproductive ability, surviving adults were generally weak unfit individuals.

The pre-adult fitness loss of *line 66* that had no detectable *omega-n* could be due to the absence of this transcript in the prothoracic gland of larvae where it occurs at high levels (Mutsuddi and Lakhotia 1995). During larval development, this is the site of production for ecdysone, the major *Drosophila* larval moulting hormone that is required for developmental growth transitions (Berreur et al. 1979). It is possible that the previously described indirect interactions between *omega-n* and ecdysone production (Ashburner 1967; Lakhotia and Singh 1982) are crucial for normal pre-adult development, particularly for the transition between pre-adult stages—this interaction being missing in *line 66*.

Since recent data suggest that natural variation in levels of *omega-n* transcript influence basal rates of general protein synthesis (Johnson et al. 2009a), we examined protein synthesis in lines with disrupted *hsr-omega* expression. While we used ovaries from gravid females for convenience, and for tissue uniformity, transcript levels were measured in RNA extracts from whole adult females. We thought this not unreasonable since a large percentage of RNA extracted from gravid females will be ovarian derived and high levels of *omega-n* normally occur in ovarian nurse and follicle cells (Mutsuddi and Lakhotia 1995). We were also concerned that the ‘normal’ progression of oogenesis in the mutants could be different than the controls and affect levels of total protein synthesis measured at any particular age, despite the fact that these mutant females were healthy and fertile. To minimise such an effect we adjusted our measure of protein synthesis for differences in ovary size across lines. Thus, our test of this cellular model of *omega-n* function has been indirect; we have not studied a uniform tissue or cell system. Nonetheless, a reduction in *hsr-omega* expression led to the predicted increase in rates of ovarian protein synthesis. The result is consistent with the working model of *omega-n* function, whereby *omega-n* sequesters pre-messenger RNA splicing factors away from pre-mRNA processing activity (Prasanth et al. 2000) thus delaying production of mature mRNA for export and translation, and limiting levels of protein synthesis (Johnson et al. 2009a). Since *omega-n* shows a widespread and general tissue distribution (Lakhotia et al. 2001) we postulate that constitutive levels of *omega-n* may set an upper limit to protein synthesis that occurs in most adult tissues of unstressed *Drosophila*—a hypothesis that needs to be tested at both the cellular and tissue levels.

While we were only effective in testing heat hardening at the higher stress temperature it is clear that the deleted lines successfully heat hardened. Therefore, ‘normal’ expression levels of *hsr-omega* appear not to be an

essential part of the heat hardening mechanism. Heat hardening capacity in *D. melanogaster* has recently been negatively associated with protein synthesis rates measured immediately following a mild heat shock (Johnson et al. 2009b), suggesting a link between the control of protein synthesis and heat hardening. While these *hsr-omega* mutations have had an effect on basal rates of protein synthesis in ovaries we have not detected an effect on adult fly hardening capacity.

The current data indicate that any effect of *hsr-omega* on adult heat-knockdown time, a specific measure of heat tolerance, is subtle and/or sex-specific. A small effect of *hsr-omega* in protection from heat paralysis was suggested by the increased knockdown time detected in mutant females in the less severe 37°C test, but not in mutant males. An overlapping but unique genetic architecture in each sex has been previously reported to underlie resistance to high temperature extremes in *D. melanogaster* (Morgan and Mackay 2006). Also, different measures of heat resistance can be surprisingly unrelated, both across strains and between sexes (Hoffmann et al. 1997), so a difference between sexes in this measure is not surprising.

In our more severe heat-knockdown tests at 39°C, no effects of the mutations were detected. Could *hsr-omega* affect heat-knockdown tolerance only at lower heat-shock temperatures? A functional *hsr-omega* could be more relevant in the 37°C test when individuals took about 80 min to knockdown (compared to ~20 min in the 39°C test). If body temperature is increased quickly, there may be insufficient time for a protective cellular heat-stress response to be implemented, as has been established for *Drosophila* cells in cultures above 37°C (Lindquist 1980). Small differences in exposure conditions can alter relevant underlying gene expression patterns (Krebs and Feder 1997; Sørensen et al. 2005; Stephanou et al. 1983) and result in different effects on any particular measure of heat tolerance. Slower heating conditions would be more relevant in natural populations on hot days when ambient temperatures increase slowly to stressful levels (Sgrò et al. 2010). However, it is possible that *hsr-omega* has no effect on heat-knockdown time, and the genotype associations detected in similar knockdown tests (Anderson et al. 2003; Rako et al. 2007) are due to effects of genes in linkage disequilibrium with *hsr-omega*, such as those within *In(3R)P* (although the tolerance associations in Rako et al. (2007) were independent of *In(3R)P*).

What heat-related fitness components might be affected by *hsr-omega* expression? The strongest evidence for a role of *hsr-omega* in heat tolerance comes from work on heat selected lines (McCull et al. 1996; McKechnie et al. 1998) where selection involved fast increase in body temperature to 39°C (resulting in a 5 min knockdown time) followed by a recovery period and breeding each generation. In these

heat-tolerant lines, a protective role of *hsr-omega* could have been during recovery rather than during the heat stress itself. Perhaps *hsr-omega* acted to delay post-heat-stress mortality, not measured in the current study. In addition, given the high abundance of *hsr-omega* transcripts in the ovaries and testes (Mutsuddi and Lakhotia 1995) it is possible that *hsr-omega* contributes to a more efficient post-stress reproductive success, such as higher fertility or fecundity. These and other more subtle heat-related fitness tests need to be examined in the mutant strains.

The *hsr-omega* gene appears to be essential for full-fitness in an optimal environment since removal of both major transcripts resulted in a 'sick' strain. However, it is not a *lethal* gene since surviving homozygous males and females are viable and reproductively capable. It is likely that *hsr-omega* is important for a healthy and competitive organism in nature where daily and sometimes harsh temperature changes occur along with other environmental stresses. Our data suggest that *hsr-omega* is not a major gene that affects two commonly used laboratory measures of heat tolerance, heat-knockdown time when abrupt changes to high body temperature are applied, and heat hardening ability. Nonetheless, the expression responses of the gene following heat stress, and the repeated spatial and environmental genotype associations, are consistent with the idea that *hsr-omega* plays a role in coping with thermal stress in natural populations. Further research should elucidate whether or not these *hsr-omega* mutants influence heat-knockdown tolerance when slow natural rates of body-temperature increase are involved, or when measurements are made on post-heat-stress survival or reproductive performance. Perhaps of greater significance is our data suggesting that one *hsr-omega* transcript acts as a negative regulator of general protein synthesis. This idea needs to be tested directly at the molecular/cellular level, and if it proves to be correct *hsr-omega* modulation of protein synthesis could help facilitate changes in thermal tolerance.

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