RESEARCH ARTICLE

Cellular damage as induced by high temperature is dependent on rate of temperature change – investigating consequences of ramping rates on molecular and organismal phenotypes in *Drosophila melanogaster*

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SUMMARY

Ecological relevance and repeatability of results obtained in different laboratories are key issues when assessing thermal tolerance of ectotherms. Traditionally, assays have used acute exposures to extreme temperatures. The outcomes of ecologically more relevant ramping experiments, however, are dependent on the rate of temperature change leading to uncertainty of the causal factor for loss of function. Here, we test the physiological consequences of exposing female *Drosophila melanogaster* to gradually increasing temperatures in so-called ramping assays. We exposed flies to ramping at rates of 0.06 and 0.1°C min⁻¹, respectively. Flies were sampled from the two treatments at 28, 30, 32, 34, 36 and 38°C and tested for heat tolerance and expression levels of the heat shock genes *hsp23* and *hsp70*, as well as Hsp70 protein. Heat shock genes were upregulated more with a slow compared with a faster ramping rate, and heat knock-down tolerance was higher in flies exposed to the faster rate. The fact that slow ramping induces a stronger stress response (Hsp expression) compared with faster ramping suggests that slow ramping induces more heat damage at the cellular level due to longer exposure time. This is supported by the observation that fast ramped flies have higher heat knock-down tolerance. Thus we observed both accumulation of thermal damage at the molecular level and heat hardening at the phenotypic level as a consequence of heat exposure. The balance between these processes is dependent on ramping rate leading to the observed variation in thermal tolerance when using different rates.

INTRODUCTION

Studies of fitness traits in insects have often revealed quantitative and qualitative differences in performance of organisms between laboratories or between laboratory and field conditions (Ackermann et al., 2001; Kristensen et al., 2008). Thus reliable quantification of fitness components requires the development of laboratory assays that are robust as well as ecologically relevant, enabling extrapolation of results to natural conditions and comparisons of results from studies performed in different laboratories. Obviously, ecological relevance is of fundamental importance if we want to use laboratory studies to investigate natural adaptation, or forecast how ectotherms will respond to temperature changes in nature. From a climate change perspective, little knowledge is gained about thermal adaptation if the methods used in the laboratory have little relation to thermal conditions in nature. Likewise, studies aiming at identifying candidate genes and mechanisms that explain variation in thermal resistance may not identify causal genes or mechanisms if test conditions in the laboratory are not ecologically relevant.

Traditional methods of assessing resistance to thermal stress involve acute exposure to high or low temperatures (for a review, see Hoffmann et al., 2003). Such assays may not be ecologically relevant because insects are almost never exposed to such drastic temperature changes in the field and because these protocols may not allow time to respond to temperature changes *via* hardening or acclimation

responses (Terblanche et al., 2006; Terblanche et al., 2008). Recently, it has been suggested that thermal assays where temperatures are gradually ramped up or down until insects lose consciousness provide better estimates of thermal maxima and thermal minima compared with traditional methods (Terblanche et al., 2007; Overgaard et al., 2012). However, methodology including the choice of rate of temperature change has a profound effect on estimates of absolute values of critical thermal limits. This has sparked a debate on the underlying causes for, and interpretation of, this variation (Rezende et al., 2011; Santos et al., 2011; Terblanche et al., 2011; Overgaard et al., 2012). A better understanding of the role of methodology will probably yield a better understanding of temperature limits and field responses to environmental temperatures in arthropods (e.g. Terblanche et al., 2007; Overgaard et al., 2011; Santos et al., 2011).

In numerous studies using thermal ramping assays to assess critical thermal maximum temperatures (CT_{max}) it has been shown that CT_{max} is higher with fast compared with slower ramping temperatures (e.g. 0.1 *vs* 0.06°C min⁻¹) (Mora and Maya, 2006; Terblanche et al., 2007; Chown et al., 2009; Overgaard et al., 2012). Rezende et al. (Rezende et al., 2011) commented on this observation and stated that it is '...a puzzling result because slower heating rates should allow individuals to acclimatize to new temperatures and because slow heating pre-exposes individuals to non-lethal high temperatures ('hardening'), which increases heat shock resistance

(see e.g. Hoffmann et al., 2003)'. However, while leaving more time to activate the heat stress response with slow ramping, slow ramping also increases the exposure time to stressful temperatures (it simply takes longer to get to a given stressful temperature at slow compared with faster ramping). Thus the balance between costs induced by longer exposure time and the potential benefits by leaving more time to induce protective mechanisms might be influenced by ramping rate.

A number of genes, transcripts, proteins and metabolites associated with heat hardening and tolerance within and across insect (especially *Drosophila*) species and populations have been identified (Hoffmann et al., 2003; Michaud et al., 2008; Ayroles et al., 2009). Hsps and especially Hsp70 have been heavily investigated in insects and many other groups of animals and plants. They are evolutionarily highly conserved and their importance in relation to coping with heat is well documented (Sørensen et al., 2003). Cellular damage, e.g. induced by heat stress, leads to a strong upregulation of most heat shock proteins and it has been suggested that the level of Hsp70 (and other Hsps) is an indicator of the amount of stress that an organism perceives (Iwama et al., 2004; Herring and Gawlik, 2007; Sørensen, 2010). Furthermore, different assays used to assess heat resistance do not always reveal the same results and the underlying physiological mechanisms might be partly or fully decoupled (Nielsen et al., 2005; Jensen et al., 2010; Mitchell and Hoffmann, 2010). Thus it is of crucial importance that we better understand what we actually measure when using different approaches to assess thermal resistance in ectotherms, and what mechanisms are involved.

In this study we tested female *Drosophila melanogaster* Meigen 1930 exposed to ramping temperatures of 0.06 or 0.1°C increases per minute starting at 25°C. Flies were collected from the two ramping rates at different temperatures (28, 30, 32, 34, 36 and 38°C) and tested for gene (*hsp23* and *hsp70*) and protein (Hsp70) expression patterns and acute heat resistance using a heat knock-down assay. Our key point was to describe physiological responses to different ramping rates and to provide information on the physiological background for the commonly observed divergence in measurements of thermal tolerance (CT_{max}) using different ramping rates. Furthermore, we provide results of general interest for the basic understanding of physiological adaptation to high temperature in ectotherms, which may also contribute to an ongoing debate about how best to measure thermal resistance in laboratory assays (Rezende et al., 2011; Santos et al., 2011; Terblanche et al., 2011; Overgaard et al., 2012). We test the following hypotheses: (1) at a given temperature, slow compared with fast ramping leads to the accumulation of more cellular damage and following this increased *hsp*/Hsp expression; (2) at a given temperature, slow compared with fast ramping leads to an increased hardening response, due to more time being available to induce the cellular stress response and the accompanying increased cumulative level of induced Hsps; and (3) the impact of variation in ramping rate on thermal resistance can be determined by the net balance of opposing protective and damage accumulating processes associated with different ramping rates.

MATERIALS AND METHODS
Maintenance and origin of experimental flies
For the experiments we used a control population of *Drosophila melanogaster* maintained in the laboratory [line C1, described in Bubliy and Loeschcke (Bubliy and Loeschcke, 2005)]. All experimental flies developed under controlled density (50 eggs per vial with 7 ml of standard fly food). Vials were provided with filter paper to provide a surface for larvae to pupate. Upon emergence of adults, flies were transferred to vials with fresh food. On the second day after emergence the flies were sorted according to gender, and females were kept in fresh vials at a density of 50 females per vial. The experiments were carried out on 4- to 5-day-old female flies.

Thermal ramping
The experimental flies were subjected to one of three treatments, either to one out of two ramping regimes (0.06 or 0.1°C increase per minute) or kept at 25°C as controls. All flies were transferred individually to screw-cap glass vials (5 ml) before being transferred to temperature-controlled water baths at 25°C. For controls the bath remained at this temperature, while the remaining water baths were subjected to an increased temperature (ramp) of 0.06 or 0.1°C min^{-1}, respectively.

A set of flies were harvested when the temperature of the ramping reached 28, 30, 32, 34, 36 and 38°C at both ramping rates, respectively (see Fig. 1). Control flies were harvested at the same time as the flies exposed to the slower ramping regime (0.06°C min^{-1}) reached the respective temperatures of 28, 30, 32, 34, 36 and 38°C.

Thermal tolerance phenotype
In an acute heat tolerance test, 20 vials of ramped flies were sampled at 28, 30, 32, 34, 36 and 38°C from each of the two ramping regimes and compared with 20 vials of control flies (sampled from 25°C). Vials of flies were immediately transferred to a rack in a transparent temperature-controlled water tank set at 39.3°C to score knock-down time. Flies were continuously monitored until they were completely immobile and the time taken to reach this point was noted. The control group was included for each ramping temperature to account for variation in treatments. The temperature was kept high and the time to knock-down therefore low (mean: 20 min, maximum: 33 min) to achieve an as acute test as possible while still maintaining a good resolution among treatment groups.

Gene and protein expression
Flies intended for molecular investigation were flash frozen in liquid nitrogen and subsequently kept at −80°C until analysis. For both protein (three replicates) and mRNA quantification (four replicates), each assayed replicate consisted of flies from 10 randomly selected vials of the same treatment. Controls were included for each time point of slow ramping to investigate if time of treatment (handling, time in vials, etc.) had an effect on the flies. Control samples for 28 and 38°C were lost during preparation. However, for no genes did the remaining controls show any tendency to change expression levels with time between sampling times. Thus from the remaining controls we conclude that the treatment, time and handling had no effect on gene expression.

For the analysis of inducible Hsp70, samples of 10 female flies were homogenized and the Hsp70 level was determined by the ELISA technique using the antibody 7FB, which is specific for the inducible Hsp70 in *D. melanogaster* (Welte et al., 1993). For each experimental condition we measured the Hsp70 expression level in quadruplicate for each of three replicate samples on ELISA micro-well plates, with one replicate for all conditions per plate (i.e. in each of three assays, one replicate of all experimental conditions was found). All assays were grand mean normalized to assay number one to correct for block effects. The ELISA procedure used is described in detail by Sørensen et al. (Sørensen et al., 1999).

For gene expression, the total RNA was extracted from macerated adult female flies using the NucleoSpin RNA II (Macherey-Nagel, Düren, Germany) with on-column DNase treatment according to the manufacturer's instructions. The samples were macerated with a steel bead in lysis buffer in 2 ml Eppendorf vials using a TissueLyser
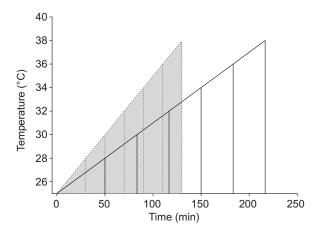
Fig. 1. Relationship between time taken and temperature reached at different ramping rates (0.06 and 0.1°C min^{-1}), starting at 25°C. Accumulated heat exposure estimated as the area under the curve is indicated for the sampling temperatures used in this study (28, 30, 32, 34, 36 and 38°C). The grey shading and dotted lines represent fast ramping (0.1°C min^{-1}) while the white area and continuous lines represent slow ramping (0.06°C min^{-1}).

II (Qiagen, Copenhagen, Denmark) at 30 Hz for two periods of 15 s. The concentration of RNA was determined spectrophotometrically using an Implen NanoPhotometer (AH Diagnostics, Aarhus, Denmark). Agarose (1%) gel electrophoresis was used to verify the quality of the RNA. cDNA was synthesized from 1.2 μg total RNA using the Omniscript Reverse Transcriptase kit (Qiagen) following the manufacturer's instructions and Anchored Oligo(dT)$_{20}$ primers (Invitrogen A/S, Taastrup, Denmark). Subsequently, cDNA was diluted to a concentration equivalent to 4 ng total RNA μl^{-1}, and stored at −20°C.

The sequences used to design primers were obtained from GenBank (http://www.ncbi.nlm.nih.gov/genbank) or primers were obtained from published literature (Bettencourt et al., 2008). Accession numbers and primer sequences for the target and reference genes are listed in Table 1. Primers were designed using Primer3 (Rozen and Skaletsky, 2000) and were synthesized by MWG (Ebersberg, Germany). Real-time quantitative polymerase chain reaction (qPCR) was conducted on a Stratagene MX3005P (AH Diagnostics) using Stratagene Brilliant II SYBR Green QPCR Mastermix (AH Diagnostics). Each reaction was run in duplicate and contained 5 μl of cDNA template (equivalent to 20 ng total RNA) along with 900 nmol l^{-1} primers in a final volume of 15 μl. The amplification was performed under the following conditions: 95°C for 10 min to activate the DNA polymerase, then 40 cycles of 95°C for 10 s and 60°C for 60 s. Melting curves were visually inspected to verify a single amplification product with no primer-dimers and the few unsuccessful runs were removed from the analyses.

Statistics

The raw qPCR data were analysed with Data Analysis for Real-Time PCR (DART-PCR) (Peirson et al., 2003). This Excel workbook enables calculation of threshold cycles and amplification efficiencies for every sample. Calculated efficiencies indicated around 2-fold amplification per PCR cycle for both genes. Differences in amplification efficiency were assessed using one-way ANOVA, based upon the null hypotheses: (i) that amplification efficiency is comparable within sample groups (outlier detection), and (ii) that amplification efficiency is comparable between sample groups (amplification equivalence) (Peirson et al., 2003). Outliers were omitted prior to further analysis in the few cases where they were identified by DART-PCR. Following analyses by DART-PCR, gene expression of *hsp23* and *hsp70* was normalized to that of *alpha-tubulin* (see Table 1). This gene was considered as a good reference gene as the expression of *alpha-tubulin* was shown to be unaffected by heat in *D. melanogaster* for at least 4 h after heat exposure (Sørensen et al., 2005). This was verified as results from the ANOVA (on log$_{10}$-transformed data to achieve normality and equal variances) showed no significant effect of treatment groups (slow ramping, fast ramping and controls) (P=0.993), temperatures (only 30 to 36°C included due to the loss of 28 and 38°C controls) (P=0.624) or the interaction between them (P=0.867).

RESULTS
Gene expression
hsp23

Data were square-root transformed to achieve normality and equal variances between temperatures and ramping rates. Two-way ANOVA on *hsp23* data showed a significant effect of ramping rate ($F_{1,22}$=12.8, P=0.002), temperature ($F_{5,22}$=162.0, P<0.001) and the interaction between them ($F_{5,22}$=3.23, P=0.025). Expression levels increased with temperatures and more so in the slowly ramped treatment, causing the significant effect of both factors and the interaction between ramping rate and temperature. *Post hoc* comparisons (Tukey's test) showed significantly higher *hsp23* levels in the slow ramping group at 36°C (P=0.012) and 38°C (P<0.001; Fig. 2A).

hsp70

Data were square-root transformed to achieve normality and equal variances between temperatures and ramping rates. Two-way ANOVA on *hsp70* data showed a significant effect of ramping rate ($F_{1,33}$=7.9, P=0.008) and temperature ($F_{5,33}$=63.2, P<0.001) but no interaction between them ($F_{5,33}$=0.98, P=0.446). Expression levels increased with temperature for both slow and fast ramping groups and expression levels in slowly ramped flies were generally higher than in fast ramped flies (Fig. 2B). However, due to the generally high variation between replicates within temperatures, *post hoc* comparisons (Tukey's test) showed no significant difference in

Table 1. Expressed sequence tag clone ID and primer sequences used for genes investigated by quantitive PCR

Gene name	Symbol	Accession no.	Primer (5′–3′)	Amplicon size (bp)
heat shock protein 70	*hsp70Aa*	FBtr0082512	ATCATGACCAAGATGCATCAGCA TTAGTCGACCTCCTCGAC	123
heat shock protein 23	*hsp23*	FBgn0001224	CGAGAGATGCCCTGCATTAT (*) CAGGACACCCTTAATGGCTA (*)	111
alpha tubulin at 84B	*aTub84B*	FBtr0081639	CCTCATAGCCGGCAGTTCGAACGT GAGCTCCCAGCAGGCGTTTCC	187

*Primers from Bettencourt et al. (Bettencourt et al., 2008). F, forward (5′–3′); R, reverse (5′–3′).

hsp70 expression between ramping rates at the individual temperatures.

Protein expression – Hsp70

Two-way ANOVA on Hsp70 data showed a significant effect of ramping rate ($F_{1,35}=7.5$, $P=0.011$) and temperature ($F_{5,35}=260.2$, $P<0.001$) but no interaction between them ($F_{5,35}=1.6$, $P=0.192$). Protein expression levels increased with temperature for both slow and fast ramping groups and protein levels in slowly ramped flies were generally higher than in fast ramped flies (Fig. 2C). *Post hoc* comparisons (Tukey's test) showed significantly higher Hsp70 levels in the slow ramping group at 34°C ($P=0.008$) and 36°C ($P=0.01$; Fig. 2C).

Heat knock-down resistance

Heat knock-down data for flies from each temperature (28, 30, 32, 34, 36 and 38°C) and ramping rate are presented relative to the value of the control flies taken from 25°C. Thus at each temperature the heat knock-down time of each fly assessed is presented relative to the average value of the 20 control flies exposed to 25°C prior to the acute heat stress exposure. The results from the two-way ANOVA showed a significant effect of ramping rate ($F_{1,237}=7.1$, $P=0.008$) and temperature ($F_{5,237}=30.3$, $P<0.001$) but no interaction between them ($F_{5,237}=0.80$, $P=0.58$). Heat knock-down time (i.e. thermal tolerance) increased with temperature for both slow and

fast ramping groups until 32°C. *Post hoc* comparisons (Tukey's test) showed significantly higher heat knock-down levels in the fast ramping group at 36°C ($P=0.005$; Fig. 2D).

DISCUSSION

It is well established in the literature that the temperature at which insects are knocked out by heat (CT_{max}) in a ramping assay depends on the ramping rate. In several species including *D. melanogaster*, it has been demonstrated that CT_{max} is higher at faster compared with slower ramping rates (Mora and Maya, 2006; Terblanche et al., 2007; Chown et al., 2009; Overgaard et al., 2012). The effect of ramping rate has posed two related questions on the use and interpretation of temperature ramping assays. The first question relates to the underlying reasons for the variation in CT_{max}, and the second question relates to the issue of which assay then measures the 'true' CT_{max}. However, the physiological consequences of different ramping rates in relation to acclimation/hardening during ramping have so far not been investigated.

Our data suggest that slow compared with fast ramping leads to accumulation of more cellular damage, and following this increased *hsp*/Hsp expression. Our results also show that although with slow ramping flies have more time to induce a plastic response, the costs associated with cellular damage induced by longer exposure time to stressful temperatures are also more severe with slow compared with fast ramping. Thus the response to, and the effect of, thermal
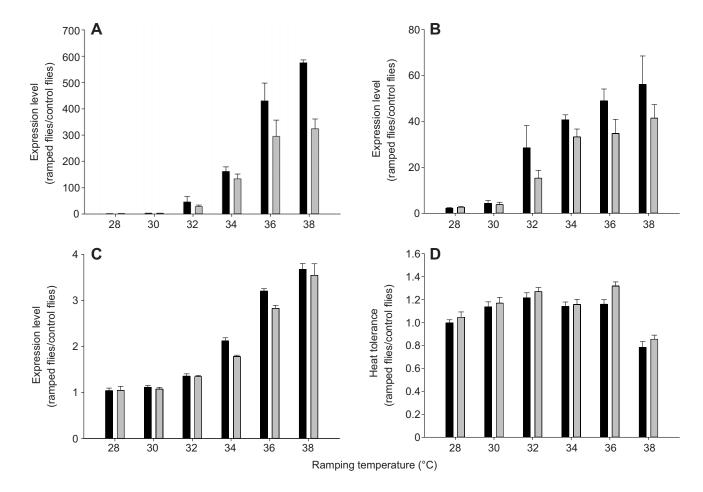

Fig. 2. Effects on female *D. melanogaster* flies exposed to thermal slow (0.06°C min⁻¹) or fast (0.1°C min⁻¹) ramping rates, represented by black and grey bars, respectively. Flies were assayed directly after reaching temperatures of 28, 30, 32, 34, 36 and 38°C, respectively. (A) Mean *hsp23* mRNA expression level (±s.e.m.) relative to the level in 25°C controls. (B) Mean *hsp70* mRNA expression level (±s.e.m.) relative to the level in 25°C controls. (C) Mean Hsp70 protein expression level (±s.e.m.) relative to the level in 25°C controls. (D) Mean heat knock-down tolerance (±s.e.m.) relative to the level in 25°C controls.

insult on the thermal tolerance phenotype is composed of two related processes. One is the accumulation of heat damage at the cellular level, which affects thermal tolerance negatively (i.e. costs). Simultaneously, hardening induced by heat exposure enhances thermal tolerance (i.e. benefits). The cost–benefit balance between these opposing forces determines thermal tolerance and is dependent on ramping rate.

The observation that heat stress markers (Hsps) were induced at a higher level at given temperatures in slow compared with fast ramping suggests that at given temperatures the slowly ramped flies have accumulated more heat damage. Adopting the simplest model for this result would be that damage is linearly related to accumulated heat exposure (a degree-time model), so that the perceived heat damage can be estimated as the area under the curve for each temperature profile ($y=0.06x+25$ and $y=0.1x+25$, respectively) (see Fig. 1). This would explain the decreased tolerance of slowly ramped flies (as the area is larger for a given temperature) and also the increased protein expression of Hsps due to the higher stress. Gene expression (mRNA) patterns of both *hsp23* and *hsp70* show similar results, and we expect that other *hsp* genes would do the same, as *hsp70* expression level is a good proxy for qualitative expression patterns of all inducible *hsp* genes (Bettencourt et al., 2008). The expression level of *hsp70* (mRNA) and Hsp70 (protein) was virtually identical. This was somewhat surprising, as a time delay in protein level is generally expected (Feder and Walser, 2005) and has been observed for *hsp70*/Hsp70, although in the springtail *Folsomia candida* and not using a ramping protocol (Bahrndorff et al., 2009). However, in our case the amount of mRNA seems to be a good proxy for the level of functional protein in the cell.

Simultaneously with inducing cellular damage, heat ramping also induced heat hardening, which increases the thermal tolerance (Fig. 2D). In this study thermal tolerance assessed using an acute heat knock-down assay was increased compared with controls up to 36°C, while the flies when ramped to 38°C had decreased tolerance compared with controls. Furthermore, while the differences were small, fast ramped flies consistently had higher tolerance than slowly ramped flies at any given temperature (Fig. 2D). Thus high temperature ramping induced two parallel dynamic processes that together determined net thermal tolerance. One is the accumulation of heat damage at the cellular level, which affected thermal tolerance in a negative way. The other related process was heat hardening, which was induced by the heat exposure and which added positively to the thermal tolerance. Both processes were affected independently by ramping rate (i.e. slower ramping leads to higher damage accumulation and increased thermal heat hardening), and we suggest that the relationship between these two processes explains the difference in absolute CT_{max} observed with different ramping rates in other studies (Mora and Maya, 2006; Terblanche et al., 2007; Chown et al., 2009; Overgaard et al., 2012). It is important to note that this result is probably species specific, and possibly affected by the heat tolerance and heat survival strategy of the species in question. For example, in the ant *Linepithema humile* (Chown et al., 2009), the beetle *Tenebrio molitor* (Allen et al., 2012) and the lepidopteran *Cydia pomonella* (Chidawanyika and Terblanche, 2011), it has been found that slow ramping rates actually increase CT_{max} compared with faster ramping rates.

If the accumulated heat exposure (estimated as the area under the curve for each temperature profile, see Fig. 1) is a valid measure of accumulated heat damage as suggested here, the expression level of stress gene expression should correlate with accumulated heat exposure, regardless of the ramping rate at which this accumulated heat exposure was achieved. Analysis of the relationship between accumulated heat exposure (area) and gene expression level for each

ramping rate did show a highly significant linear relationships (linear regression: *hsp23*, slow, $t_{15}=13.7$, $P<0.001$, $r^2=0.92$; fast, $t_{15}=9.0$, $P<0.001$, $r^2=0.83$; *hsp70*, slow, $t_{20}=7.4$, $P<0.001$, $r^2=0.72$; fast, $t_{21}=7.6$, $P<0.001$, $r^2=0.72$). Comparing slopes of the linear regressions for each gene, we could not reject the hypothesis of equal slopes among rates (parallel lines analysis: *hsp23*: $F_{1,32}=0.005$, $P=0.94$ and *hsp70*: $F_{1,44}=0.64$, $P=0.43$; Fig. 3). Thus we observe that gene expression levels of both *hsp23* and *hsp70* are increasing linearly with heat stress accumulation (area) independent of ramping rate.

Taken together, these results suggest that heat tolerance, heat hardening and heat damage can be related directly to stress level and temperature ramping rate, irrespective or independent of ramping rate. Thus under these conditions we can explain the differences in thermal tolerance as a function of rate, using only heat-related traits (heat damage and heat hardening) and without invoking other effects such as starvation and desiccation (Rezende et al., 2011; Overgaard et al., 2012). It should be noted that without a recovery period at benign temperatures after heat exposure the stress response (both induced heat tolerance and Hsp expression) was probably not fully developed. Thus if flies were allowed to recover we would probably have detected even larger differences than observed here. Yet, the more ecologically relevant conditions used still yielded potentially ecologically important effects. We argue that critical thermal limit estimates are likely to be influenced by variation from thermal history, methodology and physiological mechanisms affecting
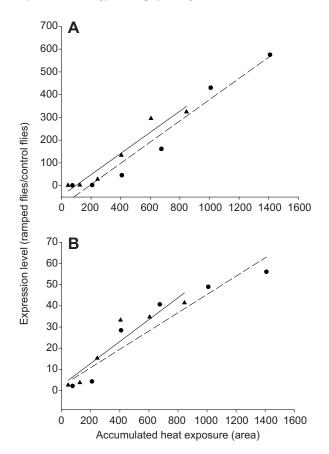

Fig. 3. Relationship between heat exposure (area, see Fig. 1) and *hsp* gene expression (mRNA) for (A) *hsp23* and (B) *hsp70* at two rates of high temperature ramping (fast: 0.1°C min^{-1}, slow: 0.06°C min^{-1}, respectively). Fast ramping is represented by the triangles and the continous line, while slow ramping is represented by the circles and the dashed line. The gene expression level is increasing with accumulated heat exposure, independent of temperature ramping rate.

absolute tolerance levels (Overgaard et al., 2012). Especially for ecologically relevant laboratory and field conditions, individuals will always carry an environmental and evolutionary history that will affect critical thermal limit estimates. Thus in our opinion the task at hand is not to identify an assay measuring 'true' critical thermal limits (CT_{max} or CT_{min}), but to understand the relationship between the environmental and evolutionary history, thermal tolerance traits and ecologically relevant assays.

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