



Biogeographic origin and thermal acclimation interact to determine survival and *hsp90* expression in *Drosophila* species submitted to thermal stress

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ABSTRACT

The relationship between thermal tolerance and environmental conditions has been extensively studied in *Drosophila*. However, comparisons of thermal tolerance of laboratory-bred flies derived from distinct geographic locations have produced puzzling results. We studied the differential expression of heat shock protein (HSP) after heat (34 °C) and cold (−4 °C) temperature treatments in two species of *Drosophila* flies, with distinct biogeographic origins (tropical = *D. melanogaster* and Andean = *D. gaucha*), previously exposed to sublethal acclimation temperatures (10, 20 and 30 °C). Also we evaluated the relationship between thermal acclimation and survival value as a proxy of fitness. We found a positive relationship between thermotolerance and the patterns of *hsp90* transcript expression in both species. Nevertheless, in the cases in which *hsp90* mRNA expression does not match thermotolerance induction, the biogeographic origin of the species could explain such mismatches. Survival at upper and lower experimental temperatures were also related with species origin.

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1. Introduction

New approaches to exploring and understanding earth ecosystems and biodiversity, have emerged with the integration of physiological ecology and macroecology, termed macrophysiology (Chown et al., 2004). In general, this convergence compares physiological features between individuals possessing different distributions (Gaston et al., 2009). Such an approach to biodiversity seeks to elucidate patterns of geographic physiological variability and to understand the mechanisms that underlie these patterns. In order to predict responses to climate change, physiological ecologists must understand the patterns of environmental variation and the mechanisms by which organisms cope with this variation. While confronting new scenarios of climate change, many organisms are expected to suffer a decrement in fitness, but some may preserve their performance through behavioral and physiological responses (Bozinovic et al., 2011). Rapid changes in environmental temperature appear as a key environmental factor influencing all aspects of organism ecology and evolution (Cossins and Bowler, 1987; Chown and Terblanche, 2007; Angilletta, 2009); and at all levels of biological organization, from molecules to organisms (Hochachka

and Somero, 2002), as well as at large geographic scales (Brown et al., 1996; Chown et al., 2002; Gaston, 2003; Chown et al., 2004; Bozinovic et al., 2011). Costs of and constraints on thermoregulation can prevent certain temperate organisms from avoiding extreme temperatures (Huey et al., 2009). Other organisms, such as tropical ectotherms, possess relatively narrow thermal limits and would suffer loss of performance when body temperature varies (Deutsch et al., 2008).

When insects are faced with temperature extremes, they may employ a range of mechanisms to maintain homeostasis, using physiological or behavioral mechanisms, or a combination of both (Chidawanyika and Terblanche, 2011). Many insect species are seasonally exposed to suboptimal or supraoptimal temperatures, and this has led to the evolution of protective biochemical and physiological mechanisms, such as the expression of heat shock proteins (HSP) (Colinet et al., 2010). Under stressful conditions, molecular chaperones stabilize denaturing proteins and refold proteins that have already been denatured, allowing the bound proteins to either attain their native conformation or be targeted for degradation and removal from the cell (Feder, 1999). In doing so, molecular chaperones minimize the probability of other proteins forming unproductive or cytotoxic aggregations.

Although stress and HSP expression are potentially major factors in determining species distribution and abundance, comparisons of the stress response in species differing in sizes of distribution ranges are scarce (Feder, 1999). Although the expression of HSPs has been widely examined for heat stress, the response to cold stress has been

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far less studied (Norry et al., 2007; Colinet et al., 2010). Exposure to heat or cold often elicits molecular and physiological responses that improve an organism's thermal tolerance. Induced thermotolerance refers to a physiological state in which organisms exposed to brief sublethal heat or cold stress acquire transient resistance to temperatures that are normally lethal (Nover, 1991; Parsell and Lindquist, 1993; Powell and Bale, 2006; Chown and Terblanche, 2007; Terblanche et al., 2007; Mitchell and Hoffmann, 2010). Previous studies indicate that HSPs are the primary mediators of induction of thermotolerance (Sorte and Hofmann, 2005; Dong and Dong, 2008).

The relationship between thermal tolerance and environmental conditions has been extensively studied in *Drosophila* (Hoffmann et al., 2003). However, comparisons of thermal tolerance of laboratory bred flies derived from distinct geographic locations have produced puzzling results. In fact, geographic comparisons of thermal tolerance traits show that differences among populations are consistent with local environmental thermal regimes (Krebs and Loeschcke, 1995; Guerra et al., 1997; Sorensen et al., 2001). However, other comparisons where latitudinal variation in thermal tolerance is expected have failed to reveal such differences (Davidson, 1990; Kimura et al., 1994). Boher et al. (2010) demonstrated that Andean *Drosophila* species do tolerate much lower temperatures than tropical ones, but thermal tolerances were not directly related to geographic distribution because there were associated fitness costs.

Present understanding of the cellular, systemic and organismal physiology of thermal homeostasis relies mainly on the response of isolated levels of biological organization. Indeed, proximal physiological mechanisms involved in large-scale patterns are poorly known (Addo-Bediako et al., 2000; Chown et al., 2002, 2004; Bozinovic et al., 2011). To shed some light on this issue, we studied the differential transcriptional expression pattern of heat shock protein 90 (*hsp90*) after heat and cold temperature treatments in two species of *Drosophila* flies, one native and one invasive, with distinct biogeographic origins, previously exposed to sublethal acclimation temperatures. We used *hsp90*, a molecular chaperone member of the heat shock protein family, which is upregulated in response not only to heat but also to cold stress (Colinet et al., 2010). We also evaluated the relationship between thermal acclimation and survival value as a proxy of fitness. In short, here we examined the impact of thermal acclimation in a native and invasive fly with different biogeographic origin and at different levels of biological organization.

2. Materials and methods

2.1. Experimental individuals

We used two species of *Drosophila* to minimize phylogenetic problems but with different biogeographic origin. *D. melanogaster* has tropical origin and is cosmopolitan (Keller, 2007), whereas *D. gaucha* exhibits a comparatively smaller geographic range with high-altitude origin in the Andes, but still inhabiting their original range (Brncic, 1969; Budnik and Brncic, 1974; Brncic, 1987). These two species coexist in nature where they exhibit similar life modes, food habits and reproductive sites (Godoy-Herrera and Connolly, 2007). We used the fourth generation of laboratory culture adults of both species to avoid potential maternal effects. Flies were reared at 24 °C in 250 mL glass vials with Burdick (1954) culture medium until the fourth generation emerged. At each generation, 40 adult flies were collected randomly from the rearing vials and transferred to fresh vials. After 3 days adults were removed to prevent overlap between generations.

2.2. Thermal treatments

To examine *hsp90* transcriptional expression, we exposed flies a few degrees above or below the lower and upper lethal limits to ensure that expression changes were not confounded by mortality. Lethal limits

were assessed by probit analysis according to Boher et al. (2010). We acclimated individuals during 1 week at 10, 20 and 30 °C (± 0.1 °C) on a 12:12 h L:D regime in climatic chambers. After that, we removed individuals from each group and randomly assigned them to upper (34 °C) and lower (-4 °C) experimental temperature tests. We transferred individuals from glass vials into plastic tubes and submerged them in a water bath with commercial antifreeze (± 0.5 °C) at the set temperature for 30 min. After upper and lower temperature tests were done, flies were returned to room temperature (24 °C). Thirty minutes after cold treatment and 15 min after heat treatment, we assessed survivorship. We considered an individual alive if it could stand on its legs after returning to room temperature. If an individual was unable to stand after this time, we considered it dead. Thereafter, only flies that were alive were frozen at -80 °C until RNA extraction. The three acclimation temperatures (10, 20 and 30 °C) were used as controls, and 34 °C and -4 °C as upper and lower thermal treatments, respectively, in *hsp90* mRNA analysis.

2.3. RNA extraction and reverse transcription

RNA extraction was performed using a filtration column, following the manufacturer's instructions (Total RNA miniprep kit, Sigma). Total RNA was eluted in 50 μ L of diethylpyrocarbonate treated water. RNA was quantified by spectrophotometry (Nanodrop 1000, Thermo Scientific), and quality-checked by running 1 μ L of each RNA sample on a 1% formaldehyde gel. Three RNA samples (i.e. biological replicates) were obtained for each thermal treatment. DNase digestion was performed to remove any potential genomic DNA contamination, using RQ1 RNase-Free Dnase (Promega), according to manufacturer's instructions. One microgram of total RNA was used in the reverse transcription to cDNA, using the Transcriptor First Strand cDNA Synthesis Kit (Roche), according to manufacturer's instructions. The cDNA was stored at -20 °C.

2.4. Real-time RT-PCR

The coding sequences of *hsp90* target gene and *rp49* reference gene from several *Drosophila* species were retrieved from the GENBANK database. PCR primers were designed from consensus sequences using Primer 3 module (<http://frodo.wi.mit.edu/primer3/>) as follows: *hsp90* forward 5'-CAAATCCCTGACCAACGACT-3', *hsp90* reverse 5'-TGATGTTG-TTGCCTTCTC-3'; *rp49* forward 5'-CACCGATTCAAGAAGTCC-3', *rp49* reverse 5'-GACGATCTCCTGCGTCT-3'. To ensure specificity of the designed primers and the proper amplification of the target gene, amplicons were cloned (pGEM-T Easy Vector Systems, Promega) and sequenced. Nucleotide sequences were compared with the US National Center for Biotechnical Information (NCBI) database using the BLAST algorithm (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>).

Real-time PCR was performed on a LightCycler 480 (Roche) system. PCR reactions were carried out in triplicate using iQ SYBR Green Super Mix (Bio-Rad), and the crossing point (C_p) was obtained using the absolute quantification module in the software package. Samples were subjected to PCR amplification with a single cycle at 95 °C for 5 min., 40 cycles at 95 °C for 30 s, 54 °C for 30 s, and 72 °C for 30 s. After the amplification phase, a dissociation curve was carried out to ensure that there was only one product. A control without template was included in all batches. The amplification efficiency of each gene was validated by constructing a standard curve through four serial dilutions of cDNA.

Data was analyzed following a method based in C_p according to the Pfaffl (2001) mathematical model, simplifying to $2^{\Delta\Delta C_t}$:

$$\Delta\Delta C_t = (C_{p,target} - C_{p,reference})_{treatment} - (C_{p,target} - C_{p,reference})_{control}$$

because the calculated efficiency values for *hsp90* and *rp49* transcript amplicons were always within the range of 95 to 100%; therefore, no correction for efficiency was used in further quantifications.

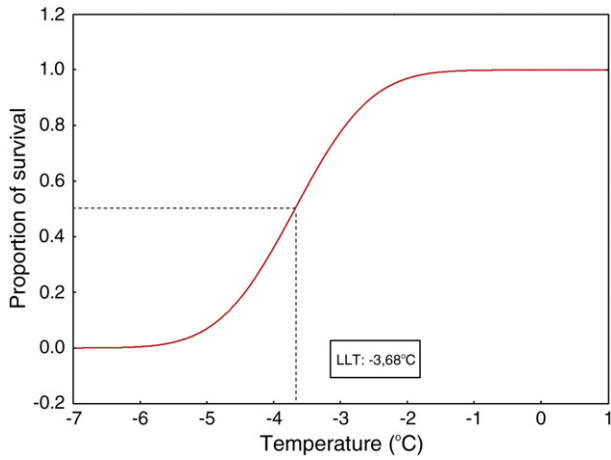


Fig. 1. Determination of LT₅₀ by probit regression analysis 7 days after acclimation to 10, 20 and 30 °C. Data in the figure corresponds to *D. melanogaster* acclimated at 30 °C. LLT: lower lethal temperature. (1344 flies).

2.5. Data analyses

We performed statistical analyses using the Statistica® (StatSoft 2001) statistical package for the Windows operating system. We used factorial ANOVA test for comparisons among fixed factors (species and acclimation temperatures). Prior to analyses we tested assumptions of normality and constant variance using Shapiro-Wilk's W and Levene's tests. Survivorship was plotted versus exposition temperature and TL₅₀ was obtained from the model using a probit analysis. Confidence intervals were estimated for each lethal temperature by Litchfield–Wilcoxon method. Statistical analysis of gene expression values was made using REST 2008 (Relative Expression Software Tool V 2.0.7; Corbett Research) (Pfaffl et al., 2002). This program calculates changes in gene expression between two groups, control and sample, using the corresponding distributions of C_p values as input. The program makes no assumptions about the distributions, evaluating the significance of the derived results by Pair-Wise Fixed Reallocation Randomization Test_tool (Pfaffl et al., 2002).

3. Results

3.1. Lethal limits

Lethal limits were obtained by probit analysis plotting temperature versus survival (Fig. 1). Upper lethal temperatures were similar in both species, but the Andean *D. gaucha* had significantly lower lethal temperatures than the invasive *D. melanogaster* (Table 1).

3.2. Thermal tolerance

Survival of both species was higher at the upper experimental temperature than at the lower experimental temperature (Fig. 2).

At the upper experimental temperature, we could not find significant differences between species ($P=0.096$; Table 2) and among acclimation treatment ($P=0.189$; Table 2). Survival at the lower thermal treatment was different for both species. *D. melanogaster* showed a marked acclimation response, i.e. a higher survival after acclimation at low temperatures, whereas *D. gaucha* had a high survival independent of the acclimation treatment (Fig. 2). There was a significant interaction between species and acclimation temperature. Thus, responses to acclimation differed between species ($P=<0.001$; Table 2). Body mass did not affect survival to upper and lower thermal treatments.

3.3. Expression of hsp90 mRNA

Transcripts encoding for *hsp90* were upregulated by heat stress in *D. gaucha* after acclimation at 10 °C (Fig. 3). Neither species showed *hsp90* mRNA over-expression in the two other upper thermal treatments (20 °C control/34 °C treatment and 30 °C control/34 °C treatment) (Fig. 3).

When flies were acclimated at 20 °C and tested at -4 °C, both species overexpressed *hsp90* mRNA relative to control although *D. melanogaster* significantly more than *D. gaucha* (Fig. 3). When flies were acclimated at 30 °C and tested at -4 °C, just *D. gaucha* showed *hsp90* mRNA upregulation (Fig. 3). It is worth noting that flies acclimated at 10 °C and tested at -4 °C do indeed have high levels of *hsp90* (5.66 and 8.48 fold change in *D. melanogaster* and *D. gaucha* respectively; Table 3), but these values were not considered significant upregulation, because control temperature has high and constitutive levels of *hsp90* expression.

4. Discussion

While induced thermotolerance is not fully understood at different levels of biological organization, a temporal relationship between *hsp90* production and thermotolerance acquisition has been reported (Parsell and Lindquist, 1993). In the present study, there were species-specific differences in the relationship between *hsp90* mRNA expression and thermotolerance. We found a positive relationship between thermotolerance and the expression patterns of transcripts encoding for *hsp90* in adults of *D. melanogaster* at low temperature treatments. However in the cases in which *hsp90* mRNA expression does not match thermotolerance induction, the biogeographic origin of the species could explain such mismatches.

We found that upper lethal limits were more conserved than lower ones, supporting previous observations (Boher et al., 2010). Indeed, Addo-Bediako et al. (2000) compiled data on both upper and lower lethal limits in insects and found that variation in upper lethal limits is much less pronounced than in lower lethal ones. These results suggest that physiological responses accounting for heat and cold tolerance are achieved by separate underlying mechanisms (Chen et al., 1990; Gaston and Chown, 1999; Chown and Nicolson, 2004). Such decoupling of upper and lower lethal limits has been documented in other species of insects (Chen et al., 1990; Kimura et al., 1994; Gaston and Chown, 1999; Addo-Bediako et al., 2000; Hercus et al., 2000; Deere et al., 2006; Ragland and Kingsolver, 2008). Lower thermal limits differed markedly between species. The temperate species with an

Table 1

Lethal temperatures in *D. melanogaster* and *D. gaucha* after acclimation for 1 week at 10, 20 and 30 °C estimated by probit analysis (Fig. 1). Different letters indicate significant differences between means.

Species	Acclimation temperature (°C)	ULT (°C)	Confidence intervals		LLT(°C)	Confidence intervals	
<i>D. melanogaster</i>	10	37.80	37.58–38.01	a	-5.05	-4.90 to -5.20	a
	20	36.69	36.47–36.92	b	-4.59	-4.34 to -4.87	b
	30	37.15	36.89–37.41	b	-3.68	-3.49 to -3.89	c
<i>D. gaucha</i>	10	36.17	35.89–36.44	c	-5.58	-4.73 to -6.60	ab
	20	35.94	35.91–35.97	c	-11.31	-10.11 to -12.65	d
	30	37.00	36.74–37.26	b	-7.85	-7.16 to -8.60	e

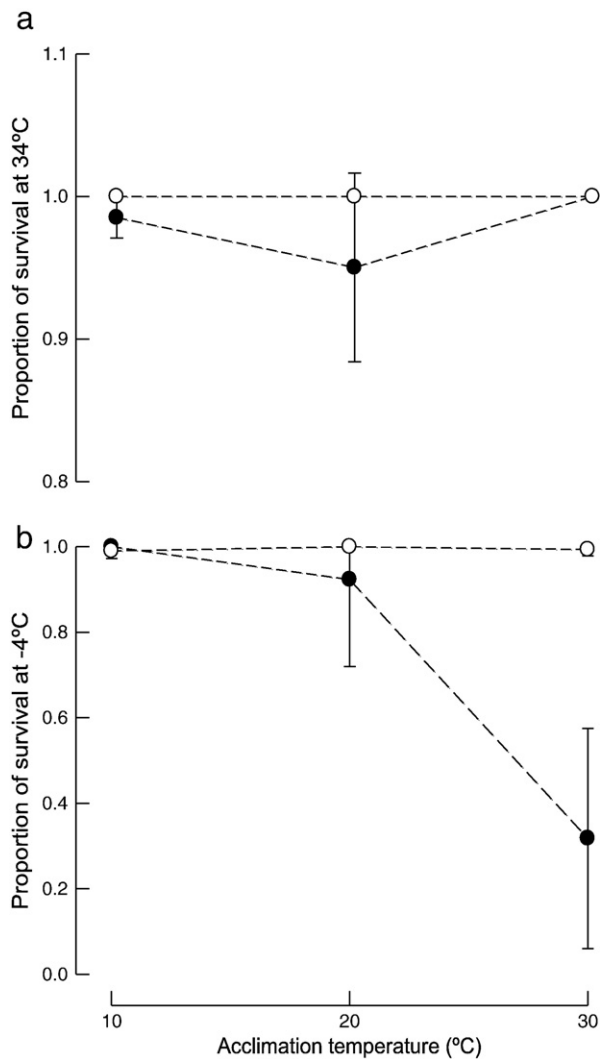


Fig. 2. Survival proportion of flies exposed for 30 min at a) 34 °C (1362 flies, 43 independent trials) and b) -4 °C (1246 flies, 38 independent trials) after a recovery time of 30 min at room temperature. Flies were acclimated during 1 week at 10, 20 and 30 °C. Data is reported as arithmetic mean \pm 1 SE. • *D. melanogaster*; o *D. gaucha*.

Andean origin (*D. gaucha*) tolerated much lower temperatures than the tropical species (*D. melanogaster*), suggesting a tendency of species to retain ancestral physiological traits.

Survival at upper and lower experimental temperatures were also related with species origin. *D. gaucha* had high survival at both temperature treatments probably because it is a species exposed to variable

Table 2

Influence of species identity, acclimation temperature and their interaction on survival at upper and lower experimental temperatures in *D. melanogaster* and *D. gaucha*.

Source	d.f.	SS	F-ratio	P
<i>Upper thermal treatment (34 °C)</i>				
Species	1	0.004	12.910	0.096
Acclimation	2	0.005	11.741	0.189
Species X acclimation	2	0.005	11.741	0.189
<i>Lower thermal treatment (-4 °C)</i>				
Species	1	0.110	13.315	0.078
Acclimation	2	0.591	18.940	0.001
Species X acclimation	2	1.763	26.650	<0.001

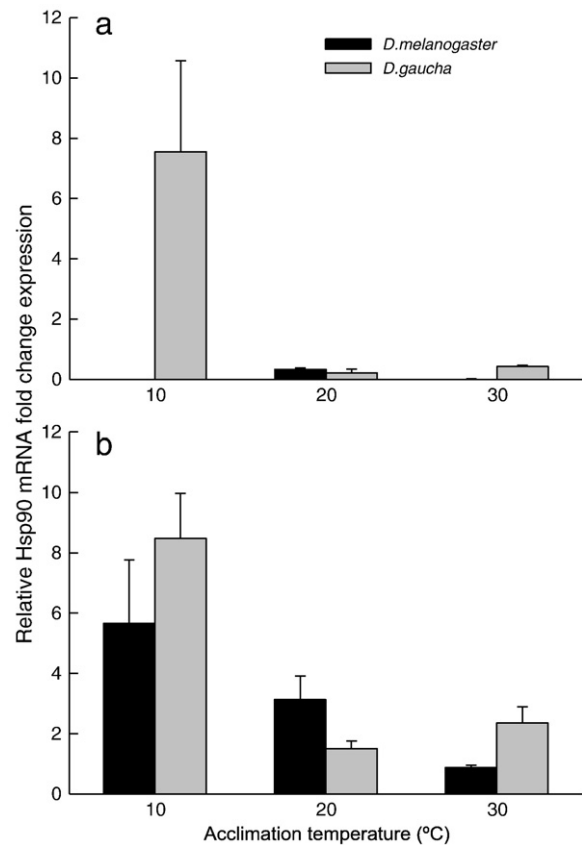


Fig. 3. Relative induction of *hsp90* mRNA in *D. melanogaster* and *D. gaucha* after acclimation at 10, 20 and 30 °C for 1 week (acclimation temperatures were used as control temperatures) in a) 34 °C sublethal thermal treatment and b) -4 °C sublethal thermal treatment (see Materials and methods). Data is reported as fold change \pm SE.

climatic conditions within its natural environment. On the other hand *D. melanogaster* had high survival at upper experimental temperatures, which is expected for a tropical species, but had lower survival at low temperature treatments with an acclimation response. Our results at the low experimental temperature support the findings of other studies done in frogs and fruit flies, in which correlations between HSP expression following sublethal shock and induced thermotolerance occur (Parsell and Lindquist, 1993; Krebs and Feder, 1998; Phang et al., 1999). Several authors have observed a correlation between surrounding temperature and acquired heat tolerance. In the olive fruit fly, *Bactrocera oleae*, (Koveos, 2001) and the leaf beetle larvae, *Chrysomela aeneicollis*, (McMillan et al., 2005) heat tolerance shows a positive association with surrounding temperatures. However this correlation was not detected here in *D. melanogaster* when tested at the upper experimental temperature. Waagner et al. (2010) did not find such correlation either in the Collembola *Folsomia candida*. Apparently, actual temperature at the time of tolerance assessment was not as important as the thermal evolutionary history. Indeed *D. melanogaster* did not show any *hsp90* mRNA expression at the upper temperature treatment at any acclimation temperature, probably because exposure at 34 °C did not represent a stressful condition to a tropical species. It has been demonstrated that a species threshold for HSP expression is correlated with the levels of thermal stress ectotherms naturally experience (Roberts et al., 1997; Tomanek and Somero, 1999; Buckley et al., 2001).

The decline in the expression of HSP in flies from laboratory strains (Lerman and Feder, 2001; *D. melanogaster* in our study at the upper experimental temperature), and from wild populations (Sorensen et

Table 3REST statistical analysis data of the expression values of *hsp90* in *D. melanogaster* and *D. gaucha* and the range of standard errors (SE) in the different thermal treatments.

Species	Acclimation temperature	Thermal treatment	Fold change	Standard error	P-value	Result
<i>D. melanogaster</i>	10 °C	34 °C	0.001	0.000–0.002	0.023	–
	20 °C	34 °C	0.329	0.287–0.382	0.031	–
	30 °C	34 °C	0.015	0.008–0.024	0.021	–
	10 °C	–4 °C	5.657	4.421–7.773	0.066	–
	20 °C	–4 °C	3.131	2.523–3.920	0.034	UP
	30 °C	–4 °C	0.871	0.803–0.952	0.024	–
<i>D. gaucha</i>	10 °C	34 °C	7.551	5.968–10.569	0.031	UP
	20 °C	34 °C	0.216	0.132–0.339	0.000	–
	30 °C	34 °C	0.424	0.384–0.466	0.000	–
	10 °C	–4 °C	8.476	7.507–9.977	0.061	–
	20 °C	–4 °C	1.509	1.259–1.747	0.023	UP
	30 °C	–4 °C	2.351	1.958–2.899	0.000	UP

al., 2001; Zatssepina et al., 2001), evolved at high temperatures, might be explained by a trade-off between costs and benefits of the expression of HSP. Constitutive resistance to high temperatures may not be maintained in individuals that do not often experience such temperatures because there are costs associated with the support of the necessary biochemical machinery (DeWitt et al., 1998; Chown and Nicolson, 2004). These strains experience most of the deleterious consequences, but derive few benefits from HSP expression because they rarely encounter potentially lethal high temperatures (Zatssepina et al., 2001). Expression of HSPs is not only associated with benefits to the organism. Continuous elevation of HSP levels results in a decline in growth and division of cells, a reduction in larvae to adult survival (Feder and Krebs, 1998; Feder, 1999) and a decline in egg hatch (Silbermann and Tatar, 2000).

Cosmopolitan *D. melanogaster* did not show any *hsp90* expression response when acclimated at 30 °C and tested at –4 °C. The lack of response is probably the responsible of the low survival when tested under the same condition. Thus, *hsp90* mRNA expression response does match the species biogeographic origin. Indeed the only species that shows an *hsp90* mRNA over-expression at the upper experimental temperature was *D. gaucha*, when acclimated at 10 °C and tested at 34 °C. Probably an Andean species did not perceive 10 °C acclimation temperature as a strong stress and when tested at 34 °C does show a markedly *hsp90* mRNA expression response to attain the high survival at this same condition. It is possible that thermotolerance in the scenarios which does not match with *hsp90* mRNA over-expression involves either another size class of HSP or a different physiological mechanism altogether.

In conclusion, although we work with laboratory bred strains of both species that were reared for four generations under the same environmental conditions, *Drosophila* species seem to retain ancestral ecological characteristics. Our results are in agreement with previous reports showing that the evolutionary history of the species may play an important role in setting their thermal tolerance limits and, thereby, their biogeographic distribution patterns (e.g., Chen et al., 1990; Guerra et al., 1997; Tomanek and Somero, 1999).

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