Ectotherms in Variable Thermal Landscapes: A Physiological Evaluation of the Invasive Potential of Fruit Flies Species

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Climate change and biological invasions pose one of the greatest threats to biodiversity. Most analyses of the potential biological impacts have focused on changes in mean temperature, but changes in thermal variance may also impact native and invasive organisms, although differentially. We assessed the combined effects of the mean and the variance of temperature on the expression of heat shock protein (\textit{hsp90}) in adults of the invasive fruit fly \textit{Drosophila melanogaster} and the native \textit{Drosophila gaucha} in Mediterranean habitats of central Chile. We observed that, under these experimental conditions, \textit{hsp90} mRNA expression was higher in the invasive species but absent in the native one. Apparently, the biogeographic origin and niche conservatism are playing a role in the heat shock response of these species under different putative scenarios of climate change. We suggest that in order to develop more realistic predictions about the biological impact of climate change and biological invasions, one must consider the interactions between the mean and variance of climatic variables, as well as the evolutionary original conditions of the native and invasive species.

Keywords: global change, environmental variability, physiological acclimation, heat shock proteins

INTRODUCTION

The question about what makes an exotic organism a successful invader has been central in the fields of applied ecology and environmental protection the last decades (Kolar and Lodge, 2001; Seastedt, 2009). Current research points out to a mix between individual and community or ecosystem features. In the community approach, focus has been on the differential characteristics of susceptible and resistant communities (Hector et al., 2001), like the hypothesis of diversity—invasiveness. At individual level the search of traits that predict the invasive potential of a species spans from reproductive potential and foraging habits to environmental tolerances (Sol et al., 2012; Bates et al., 2013; Capellini et al., 2015). It is in the later where thermal physiology provides the conceptual framework needed to explain the success—failure pattern of exotic species over the thermal landscape.

How it has been previously described (Helmuth et al., 2010; Bozinovic et al., 2011a,b; Estay et al., 2014), insights about the suitability of a thermal landscape for a given species should make reference not only to average values, but also to the intrinsic variability of
perceived temperatures (Bozinovic et al., 2016a,b). This is a key point at predicting future changes due to climate change, where theoretical (Katz et al., 2005) and empirical approaches (Easterling et al., 2000) indicate that global warming impacts not only the mean temperatures, but also the magnitude of dial and seasonal variation in temperature (Vazquez et al., 2016).

Ectotherms are particularly susceptible to temperature variation, as their body temperature is determined to a large extent by environmental conditions (Hoffmann et al., 2003; Karl et al., 2009). The ability to cope with extremes rather than different mean temperatures is probably of much greater importance for species survival and thermal adaptation (Anderson et al., 2003). The most studied physiological mechanism to cope with extreme temperatures is through the expression of stress-inducible heat-shock proteins (HSPs). These, chaperone proteins minimize the problems that arise when other proteins are in a non-native conformation (Feder, 1999; Feder and Hofmann, 1999). Most HSPs participate in protein folding and unfolding, and they are essential in cellular responses to a variety of damaging conditions (Parsell and Lindquist, 1994). Most of our knowledge about shifts in gene expression in response to changes in temperature comes from studies of the heat- and cold-shock responses (Feder and Hofmann, 1999; Johnson et al., 2009; Zhang and Denlinger, 2010). However, these studies typically focus on a narrow range of high or low temperatures that are severe and induce a strong cellular stress response. Very few studies have addressed changes in gene expression associated with routine daily or seasonal temperature regimes experienced by organisms (Podrabsky and Somero, 2004).

A simple hypothesis relating invasiveness and HSPs indicates that HSP expression is high in invasive species (Kelley, 2014); however, a new question arise: which is the baseline to compare expression levels?. An elegant solution is compare expression levels between close related non-invasive and invasive species. Some evidence pointed out that invasive species are more eurythermal than natives, i.e., have the ability to maintain physiological function over a wide range of temperatures. Unfortunately, the few studies that compared temperature tolerances between invasive and native non-invasive species have shown conflicting results (see Kelley, 2014 meta-analysis). This lack of agreement in the results could be a consequence of the importance of the evolutionary history on the current status of traits linked to thermal tolerance. In this sense, the biogeographic origin of each species is a key component in this kind of comparative analysis.

Here, we experimentally test the effects of potential scenarios of climate warming given by changes in mean temperature and thermal variance on the heat shock protein response (hsp90 mRNA) of an invasive and a native non-invasive species in central Chile. Specifically we address the following questions: (1) Does the expression of transcripts encoding for hsp90 vary across mean temperature and thermal variance treatments? (2) Does the expression of transcripts encoding for hsp90 vary between native and invasive species? and (3) How does putative climate change interact with the biogeographic origin of native and invasive species in the expression of hsp90 mRNA?

MATERIALS AND METHODS

Model Species

Our study use two species of Drosophila as a model for answering our questions: D. melanogaster and Drosophila gaucha. The former is an invasive species with a tropical origin whose range expansion may be associated with human activities (Keller, 2007). According to the entomological literature, D. melanogaster was absent from Chile until 1888 (Blanchard, 1851; Reed, 1888), and the first records from Chile can be found in the work of Sturtevant (1921). On the other hand, D. gaucha is a native species that exhibit a comparatively smaller geographic range with Andean high-altitude origin and still inhabiting in their original range (Budnik and Brncic, 1974; Brncic, 1987). Nevertheless, interestingly, these two species coexist in nature where they exhibit similar life modes, food habits and reproductive sites (Godoy-Herrera and Connolly, 2007). Adult flies were collected in Til-Til (33°05′S, 70°55′W at 586 m above sea level). The climate at this locality is Mediterranean, with an annual mean precipitation of 376 mm, concentrated 65% in winter, from June to August. Precipitation is minimal from December to March, accounting for only 3% of the yearly total. Temperatures are highest from December to March (mean = 22°C), corresponding to austral summer, and lowest from June to August (mean = 7°C), during austral winter. Boher et al. (2010, 2012) describe the range limits of both species and populations at different acclimation temperatures. In the case of D. melanogaster upper lethal limit range from 36.7 to 37.8°C, and the lower lethal limit from −5.1 to −3.7°C. On the other hand, D. gaucha showed a upper lethal limit ranging from 35.9 to 37°C, and a lower lethal limit ranging from −11.3 to −5.5°C.

Culture and Experimental Design

We used the fourth generation of field collected adults of D. melanogaster and D. gaucha to avoid potential environmental and maternal effects. Flies were reared in mass at 24°C in 250 ml glass vials with Burdick (1954) culture medium. At each generation, 40 adult flies were collected randomly from the rearing vials and transferred to fresh vials. After 3 days the adults were removed to prevent overlap between generations. Temperature range was set according to Boher et al. (2012) Drosophila thermal limits. Based on Bozinovic et al. (2011b) experimental design, during 15 days, adult flies were randomly assigned to four thermal treatments in climatic chambers; 17 ± 0°C (low mean, no variance = 17°C), 17 ± 5°C (low mean, high variance = 17°C), 24 ± 0°C (high mean, no variance = 24°C), and 24 ± 5°C (high mean, high variance = 24V). The photoperiod was L:D = 12:12h. 24°C was used as control temperature. 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). After rearing flies at constant or fluctuating temperatures, we quantified hsp90 mRNA expression in both species in each thermal scenario. Also hsp90 was the protein with more conserved alignment sequences in the primer design step. This is particularly relevant because D. gaucha, contrary to D. melanogaster, is not a model study so
conserved alignments are critical to ensure an adequate primer performance.

RNA extraction was performed on 16-day-old adults, after the 2 week acclimation period using Total RNA miniprep kit (Sigma). Each extraction was originated from a pool of 10 flies with three replicates per treatment. One microgram of total RNA was used in reverse transcription to cDNA, using the Transcriptor First Strand cDNA Synthesis Kit (Roche). Coding sequences of hsp90 target gene and rp49 housekeeping gene were retrieved from the GENBANK database. PCR primers were designed using Primer 3 module as follows: hsp90 forward 5′-CAAATCCCTGACCAAGCAGCT-3′, hsp90 reverse 5′-TGATGTGGGCGCTTCC-3′; rp49 forward 5′-CACCAGATGAAGAAGTCTTC-3′, rp49 reverse 5′-GACGATCCTCGCGCTTCT-3′.

Real-time PCR were performed on a LightCycler 480 (Roche) system. PCR reactions were carried out using iQ SYBR Green Super Mix (Bio-Rad), and the crossing point (Cp) were obtained. Samples were subjected to PCR amplification 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. A dissociation curve was carried out to ensure that there was only one product. A control without template was included in all batches. Amplification efficiency of each gene was validated by constructing a standard curve through four serial dilutions of cDNA. Data were analyzed following a method based in Cp according to Pfaffl (2001).

**Statistical Analysis**

Statistical analysis of gene expression values was carried out using the REST 2008 program (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 Cp-values as input. The program makes no assumptions about the distributions, evaluating the significance of the derived results by using the Pair-Wise Fixed Reallocation Randomization Test tool (Pfaffl et al., 2002).

**RESULTS**

All qRT-PCR assays yielded specific products (i.e., single melting peak). The acclimation temperature of 24°C was used as control in hsp90 expression analysis as was the rearing temperature for both species. The heat shock response varies greatly between the two species. *D. melanogaster* subtly increase hsp90 mRNA expression when acclimated at 24°C (*Figure 1*). The expression of hsp90 mRNA was upregulated in *D. melanogaster* after acclimation at 17°C and also after acclimation at 17V without significant differences between them (*Figure 1*).

Statistical analysis using the REST 2008 program (*Table 1*) indicates that hsp90 mRNA expression in the invasive *D. melanogaster* in both, low mean and low mean–low variance treatments, were significantly higher with respect to the other treatments and to the other species (fold change of 3.99; $P = 0.027$ and 3.13; $P = 0.048$, respectively). On the other hand, the native species, *D. gaucha* did not show hsp90 mRNA overexpression at any of our thermal treatments (*Table 1; Figure 1*).

**DISCUSSION**

Adaptation to varying thermal environments depends on the temporal pattern of environmental changes and the physiological tolerance of each phenotype (Cavieres et al., 2016). In spite of the well-known role of climate change on biodiversity (Burroughs, 2007; Angilletta, 2009; Chown et al., 2010), the range of thermal conditions in time and space, its variability and how invasive and

**TABLE 1 | REST statistical analysis data of the expression values of hsp90 and the range of standard errors (SE).**

| Species       | Thermal scenarios | Fold change | SE          | $P$-value | Result |
|---------------|-------------------|-------------|-------------|-----------|--------|
| *D. melanogaster* |                   |             |             |           |        |
| 17 vs. 24C    | 3.991             | 3.558–5.454 | 0.027       | UP        |
| 17V vs. 24C   | 3.131             | 2.612–3.670 | 0.048       | UP        |
| 24V vs. 24C   | 1.602             | 1.429–1.819 | 0.056       | –         |
| *D. gaucha*   |                   |             |             |           |        |
| 17 vs. 24C    | 0.924             | 0.746–1.138 | 0.622       | –         |
| 17V vs. 24C   | 1.035             | 0.928–1.162 | 0.751       | –         |
| 24V vs. 24C   | 1.067             | 0.890–1.260 | 0.465       | –         |

Data is representative of three biological replicates per treatment, each replicate originated from a pool of 10 flies. Data indicate that hsp90 is significantly upregulated (UP) only in individuals of the invasive species acclimated to constant and variable cold environmental conditions. Thermal treatments are: 17 ± 0°C (low mean, high variance = 17C), 17 ± 5°C (low mean, high variance = 17V), 24 ± 0°C (high mean, high variance = 24C), and 24 ± 5°C (high mean, high variance = 24V).
native animals respond to different climate change scenarios are still puzzling.

Bozinovic et al. (2013, 2016a,b) showed that those ectotherms that are continuously exposed to variations in environmental conditions deal with this variability through thermal acclimation and/or acclimatization, which impacts on the survival of natural populations. These authors also propose that if short time thermal variability changes in any of the directions forecast by climatologists, physiological approaches are necessary to predict the biodiversity consequences of climate change. In this vein, Colinet et al. (2015) showed that fluctuating ambient temperatures that remain within tolerant physiological ranges, usually improve performance in insects. Nonetheless, those which cover to extreme temperatures may have both positive impacts, allowing repair of damage accumulated to stressful conditions, or negative impacts from damage during successive exposures.

Biological invasions may interact with global warming, with invasions being favored with the increase in temperatures (Lejeune et al., 2014; Barahona-Segovia et al., 2016). Zerebecki and Sorte (2011) proposed that invasive species should be less affected by global warming than native ones. For example, native and exotic shrimp respond differentially to increasing temperatures,—the exotic species having better performance at higher temperatures. This hypothesis however was tested for aquatic species, where temperature is less variable than in terrestrial ecosystems. Barahona-Segovia et al. (2016) observed that in native and invasive ladybugs the same hypothesis is not supported, because the native species is as eurythermic as the exotic one.

To predict responses to climate change, physiological ecologists must understand the patterns of thermal variation and the mechanisms by which animals cope with this variation (Burroughs, 2007; Dillon et al., 2010). Within this framework, we experimentally assessed the likely impact of three scenarios of climate change (Burroughs, 2007) on the heat shock response of invasive and native species. Interestingly, we observed that hsp90 mRNA expression was indeed higher in the invasive species as has been reported in other studies (Henkel et al., 2009; Lockwood et al., 2010; Tomanek and Zuzow, 2010; Zerebecki and Sorte, 2011), but this species did not have a thermal range as wide as the native one (Boher et al., 2010), so we suggest that the biogeographic or evolutionary origin could be playing a role in the heat shock response of these species in different scenarios of climate change. In addition, we observed that the native species did not show hsp90 mRNA overexpression as a result of our thermal treatments. It seems that, thermically variable as well as constant environments did not represent a stressful condition in species that evolved in harsh environments such as the Andes range. Although the heat shock response is ubiquitous, it varies among species and populations in several ways including the temperature at which HSP synthesis is induced (Somero, 1995).

Comparisons of the heat shock response in species evolutionary adapted to different temperatures, as in our study, have shown that the stress needed to induce HSPs is strongly related to the realized niche of the organism in question (Feder and Hofmann, 1999). For instance, among arctic fish HSPs are induced at around 5°C (Carpenter and Hofmann, 2000) and in thermophilic bacteria at nearly 100°C (Phipps et al., 1993). Among different species of Drosophila, it was shown that expression of hsp70 is lower in lines frequently or continuously exposed to severe stress (Sorensen et al., 1999; Lansing et al., 2000). The interpretation was that the costs of HSPs expression related to fertility/fecundity, development and survival in populations frequently exposed to stress outweighed the benefits and that stress adaptation was achieved through some other means. The same pattern was subsequently found in natural populations of Drosophila (Sorensen and Loeschcke, 2001). According to these findings, the adaptive role of HSPs in connection to environmental stress resistance seems to occur during periods of relatively rare, unexpected extreme stress exposures and not during daily environmental fluctuations. On the other hand, the invasive species D. melanogaster, shows a marked heat shock response when faced to low mean and low mean-high variance treatments may be because those are stressful conditions from a species originated in tropical environments. Also D. melanogaster do present a subtle hsp90 mRNA overexpression when acclimated at high mean-high variance treatments, again possibly because, although are temperatures within their natural range, variability is perceive as a stress condition.

In conclusion, although the invasive species has the ability to express HSPs over a wider range of thermal conditions than the native species, as have been seen in other invasive-native species comparisons (Henkel et al., 2009; Lockwood et al., 2010; Zerebecki and Sorte, 2011), we suggest that the heat shock response might be also associated with the thermal history of the species, more than a “invasive ecotype” per-se (Boher et al., 2012). Indeed, many reports support a lower scope for adaptive evolutionary responses to high temperatures, meaning a more conserved heat tolerance among ectotherms in general (Boher et al., 2010; Bozinovic et al., 2014). Thus, as with upper thermal limits of tolerances, our results suggest that historical biogeography may be an important feature associated with the biochemical response of species under current and future variable climatic scenarios.

AUTHOR CONTRIBUTIONS

Conception and design: FB and FB. Acquisition and analysis: FB and NT. Drafting of the manuscript and revising it: FB, SE, and FB. All authors are approved the final version of the article.

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Parsell, D. A., and Lindquist, S. (1994). “Heat shock proteins and stress tolerance,” in The Biology of Heat Shock Proteins and Molecular Chaperones, eds R. I. Morimoto, A. Tissières, and C. Georgopoulos (Cold Spring, Harbor, NY: Cold Spring Harbor Laboratory Press), 457–494.

Pfaffl, M. W. (2001). A new mathematical model for relative quantification in real-time RT-PCR. Nucleic. Acids. Res. 29, 2002–2007. doi: 10.1093/nar/29.9.e45

Pfaffl, M. W., Horgan, G. W., and Dempfle, L. (2002). Relative Expression Software Tool (REST) for group-wise comparison and statistical analysis of relative expression results in real-time PCR. Nucleic. Acids. Res. 30:e36. doi: 10.1093/nar/30.9.e36

Phipps, B. M., Typke, D., Hegerl, R., Volker, S., Hoffmann, A., Stetter, K. O., and Baumeister, W. (1993). Structure of a molecular chaperone from a thermophilic archaebacterium. Nature 361, 475–477. doi: 10.1038/361475a0

Podrabsky, J. E., and Somero, G. N. (2004). Changes in gene expression associated with acclimation to constant temperatures and fluctuating daily temperatures in an annual killifish Austrofundulus limnaeus. J. Exp. Biol. 207, 2237–2254. doi: 10.1242/jeb.01016

Reed, E. C. (1888). Catálogo de Los Insectos Dípteros de Chile. Santiago: Anales de la Universidad de Chile LXXIII.

Seastedt, T. (2009). Ecology: traits of plant invaders. Nature 459, 783–784. doi: 10.1038/459783a

Sol, D., Maspions, J., Vall-Llosera, M., Bartomeus, I., Garcia-Peña, G. E., Piñol, J., et al. (2012). Unraveling the life history of successful invaders. Science 337, 580–583. doi: 10.1126/science.1221523

Somero, G. N. (1995). Proteins and Temperature. Annu. Rev. Physiol. 57, 43–68. doi: 10.1146/annurev.ph.57.030195.000355

Sorensen, J. G. and Loeschcke, V. (2001). Larval crowding in Drosophila melanogaster induces Hsp70 expression, and leads to increased adult longevity and adult thermal stress resistance. J. Insect Physiol. 47, 1301–1307. doi: 10.1016/S0022-1910(01)00119-6

Sorensen, J. G., Michalak, P., Justesen, J., and Loeschcke, V. (1999). Expression of the heat-shock protein Hsp70 in Drosophila buzzatii lines selected for thermal resistance. Hereditas 131, 155–164. doi: 10.1111/j.1601-5223.1999.00155.x

Sturtevant, A. H. (1921). The North American Species of Drosophila. Washington, DC: Carnegie Institution of Washington.

Tomanek, L., and Zawidzka, M. J. (2010). The proteomic response of the mussel congeners Mytilus galloprovincialis and M. trossulus to acute heat stress: implications for thermal tolerance limits and metabolic costs of thermal stress. J. Exp. Biol. 213, 3559–3574. doi: 10.1242/jeb.041228

Vazquez, D. P., Gianoli, E., Morris, W. F., and Bozinovic, F. (2016). Evolutionary and ecological impacts of increased climatic variability. Biol. Rev. Camb. Philos. Soc. doi: 10.1111/brv.12216. [Epub ahead of print].

Zerebecki, R. A., and Sorte, C. J. B. (2011). Temperature tolerance and stress proteins as mechanisms of invasive species success. PLoS ONE 6:e14806. doi: 10.1371/journal.pone.0014806

Zhang, Q., and Denlinger, D. L. (2010). Molecular characterization of heat shock protein 90, 70 and 70 cognate cDNAs and their expression patterns during thermal stress and pupal diapause in the corn earworm. J. Insect Physiol. 56, 138–150. doi: 10.1016/j.jinsphys.2009.09.013

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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