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An experimental test of the role of environmental temperature variability on ectotherm molecular, physiological and life-history traits: Implications for global warming

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ABSTRACT

Global climate change is one of the greatest threats to biodiversity; one of the most important effects is the increase in the mean earth surface temperature. However, another but poorly studied main characteristic of global change appears to be an increase in temperature variability. Most of the current analyses of global change have focused on mean values, paying less attention to the role of the fluctuations of environmental variables. We experimentally tested the effects of environmental temperature variability on characteristics associated to the fitness (body mass balance, growth rate, and survival), metabolic rate (VCO_2) and molecular traits (heat shock protein expression, Hsp70), in an ectotherm, the terrestrial woodlouse *Porcellio laevis*. Our general hypotheses are that higher values of thermal amplitude may directly affect life-history traits, increasing metabolic cost and stress responses. At first, results supported our hypotheses showing a diversity of responses among characters to the experimental thermal treatments. We emphasize that knowledge about the cellular and physiological mechanisms by which animals cope with environmental changes is essential to understand the impact of mean climatic change and variability. Also, we consider that the studies that only incorporate only mean temperatures to predict the life-history, ecological and evolutionary impact of global temperature changes present important problems to predict the diversity of responses of the organism. This is because the analysis ignores the complexity and details of the molecular and physiological processes by which animals cope with environmental variability, as well as the life-history and demographic consequences of such variability.

1. Introduction

Climate change is one of the greatest threats to biodiversity. In this sense, the Sustainable Biosphere Initiative of the Ecological Society of America recognized the importance of research on ecological consequences of global warming (Lubchenco et al., 1991). Particularly, one of the most important effects seems to be an increase in the mean surface temperature (IPCC, 2001) showing a substantial acceleration in the future (Western, 2001; Walther et al., 2002). As pointed out by Hansen et al. (2005, 2006), global surface temperature summarizes the state of global climate, and although climate effects are local, the global distribution of climate response to global climate forces is reasonably congruent with climate models, indicating that this global metric is useful. However, models (Katz et al., 2005) as well as empirical data (Easterling et al., 2000; Houghton et al., 2001) have concluded that climate change also impacts temperature variability. Indeed, thermal amplitude has been suggested to play an important selective role on life history variation (Loeschcke et al., 1997, 1999; Pétavy et al., 2001, 2004). In this regard, experimental studies testing not only the effect of increases in mean temperature but also changes in temperature variability on phenotypic traits may be important at different evolutionary, ecological, and physiological levels and scales (Travis and Futuyma, 1993; Loeschcke et al., 1997, 1999; Easterling et al., 2000; Jablonski, 2001; Houghton et al., 2001; McCarty, 2001; Pétavy et al., 2001, 2004; Inchausti and Halley, 2003; Rice and Emery, 2003; Katz et al., 2005; Folguera et al., 2008). For instance, evidence from snakes shows alterations in the ability of behavioral thermoregulation related to the thermal history of individuals (Aubret and Shine, 2010), whereas in marine invertebrates a positive relation between annual thermal variability and the molecular expression of genes sensitive to temperature has been observed (Tomanek, 2010). Over a daily scale, a decrease in the thermal sensitivity is described with the increase in the daily

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amplitude temperature (Folguera et al., 2008, 2009). Therefore, it is expected important effects over organismal traits under scenarions of environmantal temperature variability.

Understanding and explaining the selective pressures underlying differences in individual performance within species has been an important topic among evolutionary physiological ecologists. In last years, this approach was used to study the impact of climatic change in both means and variability on individual performance. Consequently, here we test the effects of daily thermal amplitude on different traits in an ectotherm, characteristics associated to the fitness (body mass balance, growth rate, and survival), physiological trait (metabolic rate (VCO_2)) and band temperature sensitive molecular traits (heat shock protein expression, Hsp70), the terrestrial woodlouse *Porcellio laevis* (Crustacea, Isopoda, and Oniscoidea). This species is a good model to test hypotheses in global change biology because its responses to thermal conditions for multiple traits are well know, and a hierarchy of physiological and life-history limits to environmental changes in time and space has been established (Castañeda et al., 2004; Lardies and Bozinovic, 2006, 2008; Folguera et al., 2009). In general terms, we predict that: 1) higher values of thermal amplitude may produce decreases in fitness of individuals, and 2) individuals submitted to higher amplitude in environmental temperature may exhibit higher metabolic cost and stress responses at molecular level.

2. Materials and methods

2.1. Capture and maintenance of Porcellio laevis

The study was performed with *P. laevis* specimens collected in the Mediterranean habitats of central Chile at San Carlos de Apoquindo (33°23′ S, 70°31′ W at 1230 m above sea level). The mean minimum temperature is 6 °C below the mean and the maximum goes 7 °C higher, on average (Jaksic, 2001). Respect to difference thorugh the year, the broadest range of temperatures is observed in Summer (16–17 °C between mean maximum and mean minimum temperature), and the narrowest in Autumm and in Winter (9–10 °C) (Jaksic 2001). After collection, organisms were sorted by sex. Pregnant females were identified in the laboratory and placed under standard conditions of light (light/dark = 12 h:12 h) and temperature (24 °C) in culture boxes (2.2 × 2.2 × 2.4 cm) with a layer of damp sand 1–1.5 cm thick.

2.2. Thermal treatments

Previuous works showed that *P. laevis* present a life cycle of about 12–18 months (Nair, 1976). At first 10 days of development, F1 were maintained at same conditions as parents at 24 °C to avoid higher rates of mortality. Then, individuals were randomly assigned to one of three thermal treatments in climatic chambers. In one of the treatments temperature was maintained at 24 °C ($\delta = 0$, δ0 group) or constant temperature regime. In the other two treatments, temperature varied along the day, reaching a maximum during the period of light and a minimum during the night (alternating temperature regimes): 32–16 °C ($\delta = 8$, δ8 group) and 28–20 °C ($\delta = 4$, δ4 group); in all cases diurnal temperature is given first. These experimental temperatures were chosen because they are within the thermal tolerance range and daily variability of *P. laevis* (Husain and Alikhan, 1979; Lardies and Bozinovic, 2006). Specimens were fed *ad libitum* during all experimental time with dry spinach. At the end of the experiments, the specimens were kept in absolute ethanol in order to be used for Hsp70 molecular studies.

2.3. Traits scored and statistical analysis

2.3.1. Body mass balance, growth rate and survival

Body mass (mb) was recorded for each woodlouse in an analytical balance (AT-21 Comparator; ±0.001 mg; Mettler, Toledo). This protocol was repeated each 14 days since 10th to 94th day (5 measurements). Standardized difference of mb (body mass balance (Δmb)) was considered as a variable: $\Delta mb = [(mb_f - mb_i)/(mb_i) \times 100] + 100$; where mb_f is mass scored in the sixth week and mb_i is that registered in the second week. Growth rate parameters were obtained from Gompertz equation (Ricklefs, 1968) of m_b through. Gompertz equation is:

$$m_{(t)} = ae^{-e^{-k(t-t_1)}}$$

where $m_{(t)}$ = body mass (mg) at age t, a = asymptotic mass (mg), k = constant growth rate ($days^{-1}$) and t_1 = age at the inflection point (days). Constant growth rate was converted to maximum growth rate (mg/days) through k times ae^{-1} (mass at the inflection point, see Zullinger et al. 1984). Survival analyses were performed using the Kaplan–Meier estimator; the contrast of the mean survival among temperature treatments was accomplished using log-rank (Mantel–Cox) for pair test. The mean and standard deviation for censoring was estimated for each thermal treatment. The survival rate was expressed as the survivor function (S(t)) as:

$$s(t) = \frac{\text{number of individuals surviving longer than } t}{\text{total number of individuals studied}}$$

2.3.2. Metabolic rate

Rates of CO_2 production (VCO_2) were determined using a "closed system" (Vleck, 1987) consisting in disposable 10 ml hermetic glass syringes fitted with three-way valves according to Lighton (2008) suggestions (see also Chappell, 1983; Chown et al., 1997). Measurements of metabolic rate (MR) were performed over adult organisms submitted to diferent thermal tratments during 100 days. Animals were weighed to the nearest mg and then placed individually within syringes. In short, each woodlouse was placed inside glass syringes that were sealed and placed in a temperature-controlled incubator at 24 °C for 3 h (measurement interval). Three blank syringes served as controls. Air was drawn from the ambient and CO_2 was scrubbed with a Drierite column. Then, the air from the syringe was slowly injected in a short Tygon tube connected to a glass tube (10 cm long) to avoid any lost before CO_2 lecture (Lighton, 2008). The air sample passed directly to the CO_2 analyzer (Fox-Box, Sable system) with a 350 ml/min flow. Data were transformed from percentage to volume per hour and the total CO_2 production per animal was calculated from the integral of the curve obtained with the program EXPEDATA (Sable Systems). This system was not intended to measure the instantaneous rate of metabolism, nor resolve discontinuous gas exchange (Chappell and Rogowitz, 2000), since each measurement is an average of CO_2 production over several hours. However, technical errors associated with this measurement method are small (Anderson et al., 1989). All animals were weighed in an analytical balance (±0.0001 g; JK-180, Chyo, Kyoto) before measurement.

2.3.3. Hsp70 molecular studies

To obtain a partial cDNA sequence of *P. laevis* Hsp70, a PCR strategy based on degenerated primer pairs was followed. The primers were designed upon conserved motifs of crustacean Hsp70 sequences present in databank. The degenerated primers were as follows: forward 5′-GAYGCVAARMTGGAYAAGGCHCA-3′; and reverse 5′-GAYTTCATGTT-GAARCARTA-3′. With them, we amplified a sequence (685 bp) that was subcloned into the pSTBlue™-1 vector (Novagen, Madison, WI, USA) and sequenced. The sequence obtained was compared by BLAST against sequences of databases and confirmed that it corresponded to a fragment of *P. laevis* Hsp70 (GenBank accession number FR693761). Expression studies of Hsp70 were carried out with quantitative real time-PCR (qRT-PCR) on specimens selected from the three experimental groups, δ0, δ4 and δ8. The efficiency of each primer was first validated by building a

standard curve through four serial dilutions of cDNA. PCR reactions were made using the IQTM SYBR Green Supermix (BioRad). Amplification reactions were carried out at 95 °C for 2 min, and 40 cycles of 95 °C for 15 s, and 60 °C for 30 s, using MyIQ Single Color Real-Time PCR Detection System (BioRad). After the amplification phase, a dissociation curve was carried out to ensure that there was only one product.

PCR specific primers were designed using the Primer Express 2.0 software (Applied Biosystems, Foster City, CA, USA) The forward primer was 5′-CAAGATTTGCTGCTGTTGGA-3′, and the reverse was 5′-GGTTTGTTTGGTGGGAATTG-3′. The U6 gene was used as a reference, and a fragment of it was amplified using the universal primers: forward 5′-CGATACAGAGAAGATTAGCATGG-3′; and reverse 5′-GTGGAACGCTT-CACGATTTT-3′. qRT-PCR reactions were performed and analyzed as previously reported (Irles et al. 2009). Total RNA was extracted from adult *P. laevis* using TRIZOL RNA Isolation Protocol (Invitrogen). Given that specimens were kept in ethanol, a washing step (Tris–HCl 0.5 M, pH 7.5 with a 2-mercaptoethanol 8%) was carried out in order to eliminate ethanol before proceeding with the extraction. A total of 400 ng of RNA from each extraction was DNAse treated (Promega, Madison, WI, USA) and reverse transcribed with the Transcriptor First Strand cDNA Synthesis Kit (Roche Applied Science) using random hexamers (Promega). RNA quantity and quality was estimated by spectrophotometric absorption at 260 nm in a Nanodrop Spectrophotometer ND-1000® (NanoDrop Technologies, Wilmington, DE, USA).

2.4. Statistics

The effects of thermal treatments on growth rate and MR were accomplished after the acclimation period. The influence of temperature variability on growth was evaluated by covariance analysis of one way (StatSoft, 2001). Response variable was considered the maximal size raise by individuals and as covariate the growth rate. The maximal size corresponds to asymptotic mass and growth rate to maximal growth rate, both estimated from Gompertz function. The influence of temperature variability on the analysis was carried out by means of a covariance analysis, with the body mass as covariate and temperature treatments as fix factor. For all analysis, the specific assumptions were evaluated previously, scaling the data to log(10) when was required. The specific contrasts among temperature treatment were accomplished by mean of the *posteriori* unequaled HSD Tukey test. All these tests were performed through the statistical package Statistica for Windows. Finally, statistical analysis of Hsp70 expression values was carried out using the REST 2008 program (Relative Expression Software Tool V 2.0.7, Corbett Research). This program makes no assumptions about the distributions, evaluating the significance of the derived results by Pair-Wise Fixed Reallocation Randomization Test tool in REST (Pfaffl et al., 2002).

3. Results

Maximum body size, growth rate and survival present similar results. Interestingly, maximum size was 12% and 23% higher at medium thermal variability (δ4 group) in comparison of maximum body size observed in δ8 and δ0 groups, respectively (Table 1). In addition, growth rate was also higher in δ4 group and lowest at the highest environmental variability, which is in δ8 group (Table 1). Survival curves (Fig. 1) revealed a clear trend where at the asymptotic 100 days survival was nearly 60% in δ8, 75% in δ4 and close to 90% in δ0 groups. Concerning physiological analyses, metabolic cost in δ8 group was a 75% higher than in δ0 and δ4 groups (Table 1).

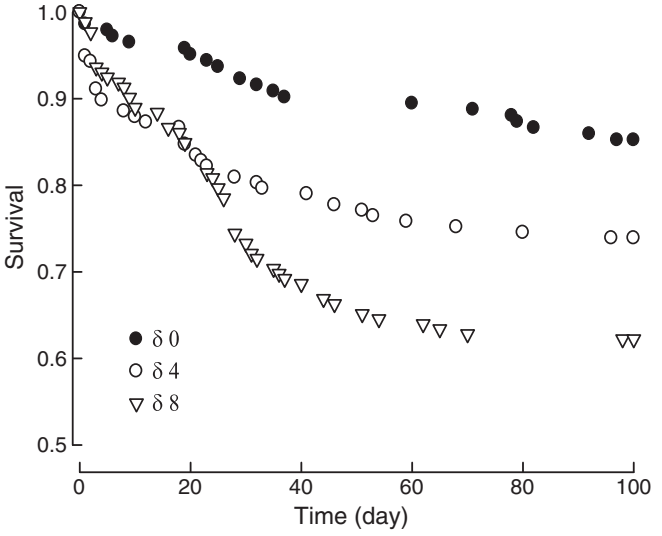

Fig. 1. Survival value in *Porcello laevis* raised in experimental environments of constant temperature (δ0 group) and temperatures varying in a range of 4 °C (δ4 group) or 8 °C (δ8 group) as a function of time.

With regard to Hsp70, Fig. 2 shows that there is a tendency to increase their expression with increasing levels of temperature variability. However, only animals raised at the highest temperature variation, the δ8 group, significantly differed either from δ0 or from δ4 groups. Statistical analysis using the REST 2008 program (Table 2) indicates that Hsp70 expression in the δ8 group was significantly higher with respect to the two other groups (fold change of 5.8 and 3.0 with respect to the δ0 and δ4 groups, respectively); between the specimens of δ0 and δ4 groups no significant differences were found.

4. Discussion

In spite of the well known role of climate change on ectotherm populations (Angilletta, 2009; Chown et al., 2010), the wide range of available thermal conditions in time and space, its variability and the thermoregulatory homeostasis of animals under different climate change scenarios are largely puzzling. In other words, physiological maintenance at cellular and individual levels, growth and survival in a given time period are dependent on mean thermal conditions and variability. Therefore, their impacts on the dynamic state-dependent

Table 1
Results after ANOVA analysis for different traits as a function of the variability in environmental temperature. Acclimation temperatures were maintained at 24 °C (δ = 0), 32–16 °C (δ = 4) and 28–20 °C (δ = 8). The ANOVA analysis for metabolic rate used body size as covariate, thus the values showed represent the adjusted means. Similar letters indicate no significant differences between means after an *a posteriori* Tukey test ($P<0.05$). In parenthesis are numbers of observations. Survival results correspond to the mean values estimate from of the non-parametric Kaplan–Meier model.

Treatments (temperature variability)					
Variable	δ = 0 °C	δ = 4 °C	δ = 8 °C		P
Hsp70 (expression × 1000 of U6/mg)	4.2 ± 2.6^{a} (18)	0.9 ± 2.4^{a} (15)	14.2 ± 2.4^{b} (21)	$F_{(2;\ 51)} = 7.46$	0.0001
Metabolic rate (ml CO_2/h)	0.001 ± 0.0001^{a} (23)	0.001 ± 0.0001^{a} (24)	0.004 ± 0.0001^{b} (18)	$F_{(2;\ 59)} = 21.08$	0.0001
Maximum size (mg)	135.8 ± 4.8^{a} (192)	176.7 ± 6.6^{b} (169)	154.0 ± 9.9^{c} (107)	$F_{(2;\ 462)} = 70.8$	0.0001
Growth rate (mg/day)	1.24 ± 0.01^{a}	1.73 ± 0.02^{b}	0.93 ± 0.02^{c}	$F_{(2;\ 462)} = 240.8$	0.0001
Survival (day)	124.9 ± 3.2^{a}	109.0 ± 4.3^{b}	96.4 ± 4.3^{c}	Log-rank (χ^2_2) = 22.8	0.0001

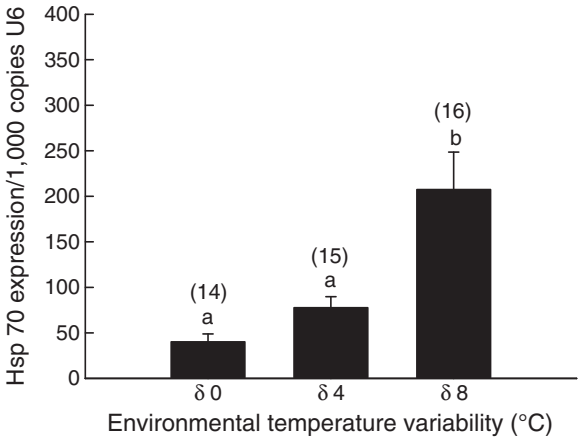

Fig. 2. Expression levels of Hsp70 with respect to U6 expression in *Porcello laevis* raised to constant temperature (δ0 group) and to temperatures varying in a range of 4 °C (δ4 group) or 8 °C (δ8 group). The number of individuals were inserted in parenthesis.

physiology should be extremely important in determining organism's life-history in a warming world. We tested the effects of thermal amplitude on characteristics related to the fitness, physiological and molecular traits and, in general terms, the results supported our hypotheses about the expected change in traits due to different thermal variability acclimation conditions. However, our results showed a diversity of responses among characters to our experimental thermal treatments.

In the case of traits related to the fitness, results were consistent with the prediction that thermal fluctuation may be an important selective factor in nature. However, the effect of thermal amplitude over whole organism responses is not trivial. In this sense, both experimental thermal extremes, — *i.e.* δ = 8 °C and δ = 0 °C, affected negatively some traits (*e.g.* maximum body size and growth rate), with respect to conditions of medium thermal variability (δ = 4 °C). From a theoretical standpoint, our results are consistent with the notion that thermal amplitude should not be necessarily considered as a stress factor, as was previously suggested (Pétavy et al., 2001). In addition, it would be expected that populations living half of the day at their optimal temperature range may have a higher reproductive rate than those living in a constant, out of the optimum lower temperature, or at least compensate for the half a day living in a lower temperature.

Heat shock proteins have an important function in cells that are under stressful conditions and they are necessary for recovery and survival of organisms, often by rescuing critical metabolic enzymes from destruction (Kregel, 2002; Sørensen et al., 2003; Dahlhoff and Rank, 2007). An organism induces various responses when it is exposed to stress, *i.e.* the repair of deoxyribonucleic acid (DNA) and protein damage, cell cycle arrest or apoptosis, the removal of cellular and molecular debris generated by stress, and there often occur changes in cellular metabolism that lead to the transition from a state of cellular growth to one of cellular repair (Kassahn et al., 2009). So inducing a stress response entails various costs for an organism. Expression of Hsps leads to reduced protein synthesis, increased maintenance costs and a decrease in the investment in growth and/or reproduction (Pörtner, 2002; Clarke, 2003). The reason for these costs are probably the shut-down of normal cell functions, the extensive use of energy and the toxic effects of high Hsp concentrations due to interference with normal cell function (Sørensen et al., 2003; Kassahn et al., 2009).

Laboratory experiments have shown that very small amounts of induced Hsps can have an influence on life history traits such as development, life span, fecundity and stress resistance (Sørensen et al., 2003). Therefore, Hsps can elicit severe effects on natural populations that are exposed to variable or changing environments, which can lead to consequences for the ecology and evolution of populations (Sørensen et al., 2003). Consequently, a number of authors have pointed the interest of investigating the expression of inducible Hsps at environmentally relevant temperature variations and the consequences of these variations on the physiology and ecology of the chosen models (Dahlhoff and Rank, 2007; Deutsch et al., 2008; Tomanek, 2010). One of the most abundantly expressed and highly conserved Hsps is Hsp70, which prevents irreversible aggregation of unfolded substrates by holding them in an intermediately folded state. It also catalyzes the refolding of unfolded substrates into an energy- and co-chaperone-dependent reaction (Nollen and Morimoto, 2002). Our studies have shown that Hsp70 expression tends to be higher when *P. laevis* samples were reared under higher temperature variations. The outcome of the REST analysis confirmed this tendency, and this result is in agreement with our hypothesis that Hsp70 would have the highest levels of expression in the samples of developmental conditions with the biggest variations. According to this general idea, the relative high values of Hsp70 expression in δ8 group leads to the prediction that they would be less efficient in most physiological processes, which is exactly what we observed in our experiments.

Based on the allocation principle, the energetic costs in which animals may sustain due to thermal stress – defined as an environmental factor that affect the physiological homeostasis with negative consequences on fitness – would reduce the amount of resources/energy allocated to reproduction and maintenance. In our case, a higher cost due to Hsp70 overexpression and a higher

Table 2
REST statistical analysis data (see Material and methods) of the expression values of Hsp 70 and the range of standard errors represented in Fig. 2. Data indicate that Hsp70 is upregulated (UP) in individuals of the δ8 group in comparison to those of δ0 and δ4 groups.

Groups	Fold change	Standard errors	P-value	Result
δ0 *versus* δ4	2.196	0.723–7.556	0.057	
δ4 *versus* δ8	3.030	0.876–9.673	0.002	UP
δ0 *versus* δ8	5.830	1.734–19.439	<0.0001	UP

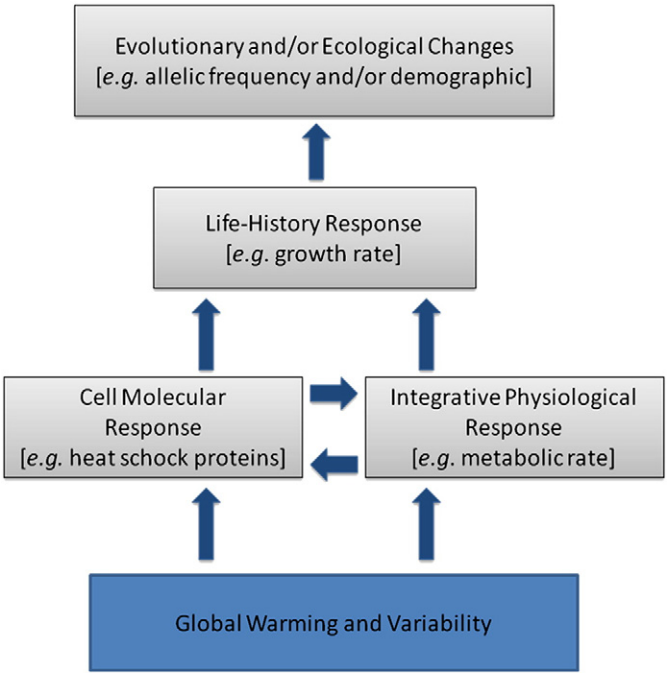

Fig. 3. Role of global warming, thermal variability, and biological features of organism (from cell to life history traits) as the determinants of ecological and evolutionary changes. We have emphasized that knowledge about the mechanism by which animals respond to environmental variability is essential to understand global climatic change.

metabolic cost of maintenance is correlated with a lower growth rate, body size and survival, apparently because a large amount of resources are used for paying the cost of maintenance. Indeed, the strong reduction in life/history investment might be the combined effect of trade-offs among different energy demanding biological processes, which followed the principle of allocation (Cody, 1966) in the sense that animals from habitats with variable temperature could have higher metabolic costs than those living in stable ones (see Pétavy et al., 2004). For example, due to the metabolic costs of acclimation, the reproductive dynamics may decrease and fitness parameters may decrease too. In this sense, inhabiting a variable temperature habitat or geographic region represents an extra cost for ectothermic animals as indicated by the concomitant higher Hsp 70 expression and metabolic costs recorded in the δ8 group.

Finally, as summarized in Fig. 3, we have emphasized that knowledge about the cellular and physiological mechanism by which animals cope with environmental changes is essential to understand the impact of mean climatic change and variability. The simple analysis of increases in mean temperatures to predict the life-history, ecological and evolutionary impact of global temperature changes is unable to predict the diversity of animal responses in many cases. Such an approach may be unsatisfactory because it ignores the complexity and details of the molecular and physiological processes by which animals cope with environmental variability, so it largely misses the life-history and demographic consequences of such variability.

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