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Plasticity of life-cycle, physiological thermal traits and *Hsp70* gene expression in an insect along the ontogeny: Effect of temperature variability

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ABSTRACT

It is considered that extreme environmental temperature, rather than mean temperatures exert a selective pressure in ectotherms. Consequently, it is important to understand how the predicted increase in temperature variance with a higher frequency of extreme events in climate change is likely to impact on organisms. Thermal tolerance traits (i.e. chill-coma, recovery time, *Hsp70* expression) are directly linked with performance in ectotherms and have consequences in life-history traits. We examined the effects of temperature variability on thermal tolerance and life-history traits through ontogeny of an insect with a complex life-cycle: the yellow mealworm beetle *Tenebrio molitor*. We established two common gardens with 100 recently ovoposited eggs each. Larvae were reared from hatching to adult on either a variable (mean = 18 °C and a variance of 6.8 °C) or constant (18 ± 1 °C) thermal environment. Development rate and growth rate were similar between thermal environments. Results indicate that larvae reared in a variable environment are more cold-tolerant than larvae of a constant environment. Interestingly, these results are reversed in the adult stage, outlining an inter-stage physiological cost. Gene expression pattern of an *Hsp70* gene was well correlated with larval thermotolerance to cold in the variable environment but higher gene expression in adults is not correlated with individual's thermotolerance. We conclude that chill-coma, recovery time and *Hsp70* gene expression are plastic in response to a thermal environment but also change significantly their responses depending on the ontogenetic stage, implying that the response of adult individuals is linked to early stages of the life-cycle.

1. Introduction

It has been shown that the thermal performance of many organisms is proportional to the magnitude of temperature variation that they experience (Addo-Bediako et al., 2002; Gilman et al., 2006). Global warming in this century is one of the largest anthropogenic disturbances placed on natural systems (Gilman et al., 2006; Walther et al., 2002). One important determinant of biological responses to climate change will be the degree of warming itself (IPCC, 2007). Also relevant, is the physiological sensitivity of organisms to changes in the temperature of their environment (Calosi et al., 2007; Helmuth et al., 2006). It is expected that global warming will modify the physiological, biochemical and ecological responses of organisms (Easterling et al., 2000; Katz et al., 2005). Therefore, an integrated view about the effects of global warming will be reached not only by establishing how the increase in mean temperature impacts the natural populations but also establishing the effects of the increase in temperature variance with a higher frequency of extreme events (see Pertoldi and Bach, 2007).

The environmentally induced phenotypic change, namely, phenotypic plasticity has been recognized as an important strategy for maximizing or maintaining fitness in an organism that inhabits variable environments (Bouvet et al., 2005; Pigliucci, 2005; Schlichting and Pigliucci, 1998; Via and Lande, 1985). In animals with complex life-cycles, such as insects, the capacity to exhibit alternative phenotypes offers an individual opportunity to optimize their fitness as they experience different environments through their lives. Significant progress has been made in identifying environmental cues that cause the induction and the range and kind of traits that are altered by different environments (West-Eberhard, 2003). Nevertheless, how the thermal environment experienced in early ontogeny affects the thermal tolerance capacities and plasticity in later instars in key traits is scarcely described in literature (Bowler and Terblanche, 2008).

It is well known that environmental temperature (T_a) is the abiotic factor with an all-pervasive influence on the physiology

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and ecology of most of biodiversity in the planet, and, this is particularly true for ectotherms (Angilletta, 2009 and references therein). This means that any performance trait (e.g. growth, reproduction, physiology) in an ectotherm will change as T_a changes (i.e. a thermal reaction norm), a relationship that has been described by a thermal performance curve (Huey and Berrigan, 2001; Angilletta, 2009). This curve is best captured by three parameters: a minimum critical temperature (CT_{min}), which represents T_a below which performance is minimum; a maximum critical temperature (CT_{max}), which represents T_a above which performance is also minimum and an optimum temperature (T_{opt}), which represents T_a at which performance is maximum. The curve rises gradually from CT_{min} to T_{opt} and then decreases gradually but rapidly to CT_{max}. Recent global analyses of both CT_{max} and CT_{min} have evidenced that both traits and tolerance breath (i.e. $CT_{max}-CT_{min}$) are affected by latitude (Deutsch et al., 2008; Huey et al., 2009; Sunday et al., 2010), suggesting at least some adaptation to T_a. A posterior estimation of the measurement of critical temperature is the "recovery time" which is the time that an individual takes to recover its mobility following chill-coma once it has been returned to room temperature (Castañeda et al., 2005). Recently, many authors have utilized recovery time from chill-coma as an estimation of cold tolerance (David et al., 2003; Hallas et al., 2002; Hoffmann et al., 2002). Recovery time has been linked to thermal tolerance due to its direct relationship with chilling duration (David et al., 1998), and identifies the tolerance to extreme events.

As mentioned before, the mean and extreme values of temperature have an important impact across every level of a biological organization, from the whole organism to the molecular level (Buckley et al., 2001; Tomanek and Somero, 2002). One of the molecular responses that is activated in a cell under temperature stress is the heat shock protein response (HSP), an event of genetic activation that occurs in the cells in response to abnormal, stressfully high or low temperatures (Hofmann, 2005). The genes that encode for Heat-Shock Proteins (HSPs) are highly conserved and have been found in every studied species (Feder and Hofmann, 1999; Yeh and Hsu, 2002). Among HSP families, the group in the 70-kDa size range (Hsp 70) is the most extensively studied because of its prominent response to stresses (see reviews in Feder and Hofmann, 1999; Sanders, 1993). The expression of these protein patterns could explain not only differences in fitness but also the geographical distribution of the organisms (Sorte and Hofmann, 2005). Recent studies in natural systems have shown that the patterns of expression in HSPs exhibit phenotypic plasticity in the thermal history of an organism (Buckley et al., 2001; Hammond and Hofmann, 2010; Osovitz and Hofmann, 2005; Tomanek and Somero, 1999). Furthermore, the synthesis, degradation and replacement of these proteins imply an increase in energetic costs of the organisms (Hartl and Hayer-Hartl, 2002; Sorensen and Loeschcke 2002). Therefore, traits trade-offs or constraint of HSP expression can be expected to be conditioned by the thermal environment experienced by early instars in the life-cycle (Hoffmann et al., 2003).

In this study, we examined the effects of temperature variability in *Hsp70* gene expression, thermal and life-cycle traits in an ectotherm with a complex life-cycle using the yellow mealworm beetle *Tenebrio molitor* (Polyphaga, Tenebrionidae), which is a cosmopolitan pest of stored grains that can be easily reared in the laboratory (Worden and Parker, 2001). *T. molitor* has been used previously in biochemical, ecological and evolutionary research (Graham et al., 2000; Drnevich et al., 2000; Vainikka et al., 2006; Worden and Parker, 2001), however, we are not aware of any studies examining traits plasticity relating with phases of ontogeny. Our study had the following three objectives: (1) to evaluate the effect of temperature variability in thermal and life-cycle traits; (2) to determine how temperature variability expresses trade-offs among traits; and (3) to quantify thermal effect through different ontogenetic stages in analyzed traits.

2. Materials and methods

2.1. Animals, maintenance and culture

The common yellow mealworm beetle *T. molitor* Linnaeus (Coleoptera: Tenebrionidae) is a species widespread through the world, which is also found throughout Chile (Vidal and Guerrero, 2007). Laboratory colony originated from individuals taken from the field at the Estación de Investigación Ecológica Mediterránea de la Pontificia Universidad Católica de Chile (EDIEM, PUC) in San Carlos de Apoquindo (http://www.bio.puc.cl/ediem/). The survival of individuals to laboratory conditions (18 ± 2 °C and photoperiod 12L:12D) was above 90% with successful reproduction and a life-cycle duration similar to reported in natural conditions. Individuals were continuosly removed from colony and new beetles were added every 2 months from the field to prevent inbreeding. In the laboratory the animals were classified by sex and ontogeny stage (Bhattacharya et al., 1970; Vidal and Guerrero, 2007) and then transferred to plastic containers and acclimated for 3 weeks at 18 ± 1 °C, with 12L:12D photoperiod. They were fed *ad libitum* with a mix of 60% wheat flour, 20% oats, 10% wheat bran and 10% brewer's yeast, this mix was immersed in a bed of wheat middlings (Martin and Hare, 1942). The beetles were mated ($n=40$ pairs) and 1 week after the eggs were obtained, these were randomly separated, individualized and reared on six well plates. Two hundred eggs were reared in each environment (see below), with a hatching percentage of 98.1%.

2.2. Thermal environments design

The acclimation was carried out in two common gardens with different environmental temperatures. The constant thermal environment had a mean temperature of 18 ± 1 °C. The variable thermal environment had a mean of 18 °C and a variance of 6.8 °C (-2.5 and 43 °C for lower and upper temperature, respectively) and with a 12 h temperature fluctuation. For the variable environment the environmental chamber was programmed weekly using random temperatures following a normal distribution from daily temperatures observed in the last 5 years (minimum and maximum mean temperatures) in central Chile. Temperature data were obtained from the weather station located at the same ecological station where beetles were collected in San Carlos de Apoquindo (for details see Jaksic, 2001). Humidity and photoperiod were maintained equal to the constant thermal environment (85% relative humidity and 12L:12D photoperiod), and the food was the same mix mentioned before throughout the complete life-cycle.

2.3. Life-cycle traits

The body mass in different environments and ontogeny stages was recorded every 14 days, starting on the 31st day of life until death using an analytical balance (ADAM AFA-180LC with precision ±0.001 mg). Relative growth rate (RGR) (van Emden, 1969) was calculated using the periodical weights of larvae using the equation: $RGR=[(mbt_1-mbt_0)/T]$; where mbt_0 is the body mass (mg) at the start of the time interval, mbt_1 is the body mass at the end of the time interval, and T is the number of days between the start (t_0) and final (t_1). We determined larval growth rate (LGR) in intervals during the life-cycle. In addition, the number of days for each ontogeny stage (larvae, pupae and adult) was recorded.

2.4. Thermal sensitivity and thermal tolerance

Each individual (i.e. larvae and adult) acclimated in constant or variable thermal environment was individualized in plastic chambers with six plates ($120 \times 80 \times 20$ mm^3) that were placed inside an incubator and maintained at the corresponding experimental temperature using a thermal bath (Labtech LCB-R20 precision ± 0.1 °C). Larval estimations of thermal tolerance and sensitivity traits were immediately determined before the sixth larval molt. Before the measurements the body mass of the beetles were recorded with an analytical balance. After 30 min of exposure to cold experimental temperatures, larvae or adult of *T. Molitor* were turned over and if an individual was not in chill-coma, it responded by returning to an upright position. If after 10 min, animals did not show coordinated movements and were still inverted, they were considered in chill-coma. Temperature was decreased by 1 °C from 10 °C until the beetle was in chill-coma. Only these animals (that had incapacity to rollover) were brought to room temperature (20 ± 2 °C) and the time that it took each individual to recover its capacity to rollover was registered and called "recovery time" (see Castañeda et al., 2004; Macdonald et al., 2004). All animals that died or did not present movements in the time for each analysis were excluded from this study. The other animals were returned to their respective environments (constant or variable), and after 2 and 4 days the seventh molt larvae were dissected to obtain their digestive tube and exoskeleton. For further analysis of *Hsp70* gene expression, animals were dissected at the same time of day and tissue was stored at –80 °C.

2.5. RNA extraction and real time RT-PCR

Total RNA was extracted separately from each larvae and adults using TrIzol® Reagent following the manufacturer's instructions. For cDNA synthesis, one microgram of total RNA was treated with DNAse I (RQ1, Promega) and then reverse transcribed with random hexamers using the ImProm-II™ Reverse Transcription System (Promega). Relative transcript quantification of isolated gene fragments was achieved by real time RT-PCR using SYBR Green Master Mix (Quantace) and the Gene-Mx3000P® detection system (Stratagene) as described in the manufacturer's manual. Briefly, the PCR mixture (25 μl) contained 2 μl of cDNA and 140 nM of each primer. Amplification was performed under the following conditions: 95 °C for 10 min, followed by 35 cycles of 94 °C, 30 s; Melting Temperature for 30 s; and 72 °C, 40 s. At the end of PCR amplification all products were subjected to a melt cycle from 55 to 95 °C. Melting temperatures were 56 and 60 °C for *Hsp70*-like and *18S* gene, respectively. Primers used were: HSP70 F: 5′ACCACCTACTCCTGCGTG3′ and HSP70R: 5′GTTCATGGCCACCTGGTTCTT3′ to amplify an amplicon of 149 pb of Hsp70 cDNA; and Tm18S F: 5′CCCGTCGCTACTACCGATTG3′ and Tm18S R: 5′CATCTTCCCAGCAACATCG′, which amplify 90 pb of 18S cDNA. 18S RNA sequence was retrieved from NCBI database (accession number X07801); amplicon of *Hsp70*-like gene correspond to a gene fragment with high similitude to *Hsp70* genes of other insects and organisms (see the alignment in Supplementary Fig. S1). Reaction specificities were tested with melting gradients dissociation curves, electrophoresis gels and cloning and sequencing of each PCR product. 25 individuals were analyzed separately (i.e. independent replicates) for each stage and environment ($N=100$). All samples were analyzed with two technical replicates. Relative gene expression calculations were conducted as described in the software manufacturer's instructions: Gene expression levels were normalized to the 18S gene expression, because the expression of this gene was well conserved in different samples and stages. Also, *Hsp70* expression level was calibrated in each sample and then calibrated to the treatment/stage of lowest expression using the $2^{-\Delta\Delta ct}$ equation.

2.6. Statistical analysis

We used one-way ANOVA to test differences in days in the different development stages between thermal treatments and to evaluate differences in body size of larvae and adults. An ANCOVA with homogeneity of slopes model was used to compare RGR in larval stage. A repeated measures ANOVA model was utilized to test the effect of thermal treatments at different times of ontogenetic development on RGR. The same analysis was used to evaluate differences in means of recovery time of larvae and adults between thermal treatments. A two-way mixed model ANOVA was used to test the effect of thermal treatments and ontogenetic stage (i.e. factors) in chill-coma temperature and *Hsp70* qPCR (i.e. responses variables). We checked assumptions of normality and homoscedasticity of raw data using Kolgomorov–Smirnov and Levene tests, respectively. Data of Hsp70-like qPCR and chill-coma temperatures were arcsine transformed to meet ANOVA assumptions. All statistical analyses were conducted using SPSS 12.0 software (Apache Software Foundation, Somers, New York), where a level $p < 0.05$ was used to reject the null hypothesis. Data are presented as mean $\pm$ standard error of the mean (S.E.M.).

3. Results

3.1. Life-cycle traits

No differences in body mass were found between beetles reared in thermal treatments of both larvae (one-way ANOVA; $F_{1,125}=1.52$, $p=0.191$) and adults (one-way ANOVA; $F_{1,82}=0.84$, $p=0.242$). The larvae acclimated in the constant and variable environments at 59 days showed an RGR mean of 0.3 ± 0.04 and 0.5 ± 0.10 mg/day, respectively. At 143 days of larvae they had an RGR mean of 1.3 ± 0.24 and 1.1 ± 0.11 mg/day for constant and variable environments, respectively (Table 1). Significant differences were not observed in RGR between thermal treatments (repeated measures ANOVA; $F_{1,73}=2.85$, $p=0.096$) but the RGR was significantly higher at 143 days compared with 59 days of larvae life (repeated measures ANOVA; $F_{1,73}=38.99$, $p < 0.0001$). Growth curve slopes were similar in both thermal treatments (homogeneity of slopes model; $F_{1,340}=2.48$, $p=0.218$).

Table 1
Number of development days and relative growth rate for each ontogeny stage of *Tenebrio molitor* acclimated since they started life in the different thermal environments (mean $\pm$ S.E.M.).

	Constant		Variable	
	Days	**Relative growth rate (mg/day)**	**Days**	**Relative growth rate (mg/day)**
Larvae	192.1 ± 16.9	1.3 ± 0.24	192.3 ± 26.4	1.1 ± 0.11
Pupae	18.2 ± 2.6	–	15.2 ± 4.3	–
Adult	24.5 ± 12.4	0.02 ± 0.08	15.9 ± 9.8	-0.03 ± 0.12

Adults RGR was almost nil and significant differences were not observed (one-way ANOVA; $F_{1,64}=0.06$, $p=0.8416$) (Table 1).

Beetles from both thermal treatments did not exhibit differences in the total number of days in larval stage (one-way ANOVA; $F_{1,66}=0.002$, $p=0.9634$) (Table 1). The mean for the number of days of adult stage was significantly longer in constant environment 24.5 ± 12.4 days compared with beetles of variable environment 15.9 ± 9.8 days (one-way ANOVA; $F_{1,64}=10.28$, $p=0.002$). The beetles' complete life-cycle in the constant environment was 235.8 ± 18.5 (mean ± S.E.M.) days and in the variable environment 217.7 ± 24.4 (mean ± S.E.M.) days showing significant differences (one-way ANOVA; $F_{1,63}=11.54$, $p=0.0011$) (Table 1). This difference in the total number of days of life-cycle is produced by shorter life in adult stage in the variable environment. Furthermore, more beetles died in the variable thermal environment where the mortality rate was 63.3% in comparison to the constant environment (11.6%).

3.2. Thermal tolerance traits

The larvae acclimated in the constant and variable thermal environments had chill-comas of 1.2 ± 0.2 and 0.5 ± 0.3 °C, respectively (Fig. 1). Chill-coma temperature in adult stage was 1.8 ± 0.2 °C in constant environment and 2.4 ± 0.2 °C in variable environment. A two-way ANOVA analysis show that chill-coma was significantly different between environments (two-way ANOVA; $F_{1,112}=4.43$, $p=0.027$). Furthermore, the interaction between ontogenetic stages and environment was significant in chill-coma response (two-way ANOVA; $F_{1,112}=20.47$, $p<0.0001$), that is the chill-coma response is higher in adult stage compared with larvae but larval response depended on the rearing environment (see Fig. 1).

Recovery time of larvae acclimated in constant and variable environments were 273.5 ± 24.4 and 283.3 ± 22.9 s, respectively (Fig. 2). Adults showed values of 462.0 ± 42.4 and 421.5 ± 67.0 s in constant and variable environments, respectively (Fig. 2). The recovery time varied as a function of the ontogenetic stage, a significant increase in the recovery time of adults in respect to larvae was observed (repeated measures ANOVA; $F_{1,40}=4.91$, $p=0.032$), independent to the rearing environment.

The relative expression of the *Hsp70*-like transcripts in the larvae acclimated in the constant environment was of 1.41 ± 0.34 and 2.15 ± 0.57 in the variable environment (Fig. 3). In the case of adults this relation was 11.87 ± 1.98 and 16.73 ± 4.54 for constant and variable environments, respectively. A two-way ANOVA to evaluate differences in *Hsp70*-like expression considering as factors, ontogenetic stage and thermal environments, showed a significant effect of both factors without significant interaction among them (two-way ANOVA; $F_{1,94}=49.06$, $p<0.0001$ and $F_{1,94}=3.98$, $p=0.048$ for ontogenetic stage and environment, respectively) (see Fig. 3).

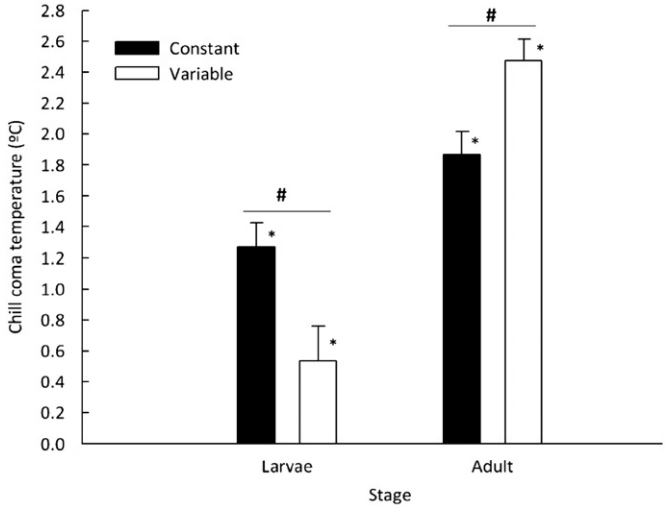

Fig. 1. Chill-coma temperature in different stages of *Tenebrio molitor* acclimated in thermal treatments. The asterisk indicates significant statistics between stages but different thermal environment and the (#) represent interaction significant amount the different factors (stage and environment). The bars indicate (mean ± S.E.M.).

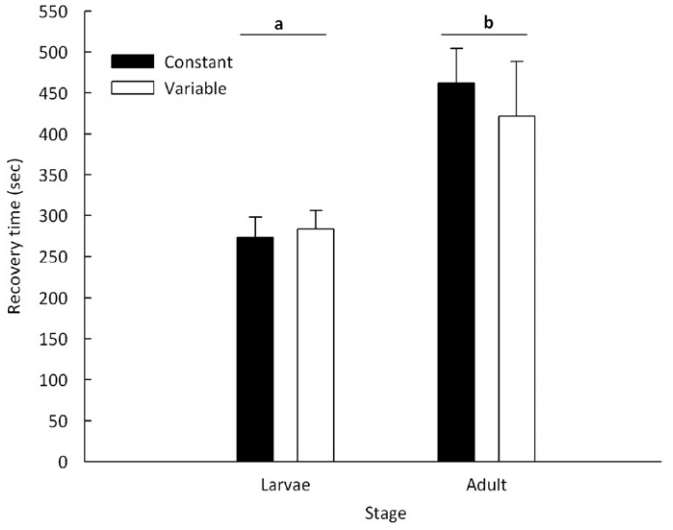

Fig. 2. Recovery time for chill-coma temperature in the different stages of *Tenebrio molitor* acclimated in the different environments. Different letters indicates significant in the recovery time between ontogenetic stages. The bars represented (mean ± S.E.M.).

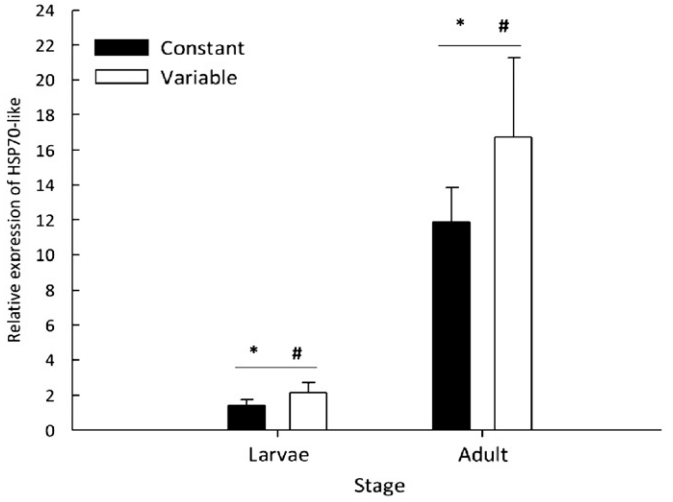

Fig. 3. *Hsp70*-like gene expression relative to 18s expression in larvae and adult of *Tenebrio molitor*. (*) Represents significant differences between stage and (#) shows significant differences between environments (mean ± S.E.M.) in *Hsp70*/*18 s* expression.

4. Discussion

4.1. Life-cycle

It is suggested that extremes of environmental variables, not their means, exert a selective pressure in ectotherms (Kingsolver and Huey, 2008; Stevens, 2004). These temperature thresholds have

profound consequences in life-history traits of ectotherms. Contrary to what would be expected, the duration of the larval stage and larval growth rates in *T. molitor* were similar among thermal treatments despite their contrasting differences in variance of temperature (see Fig. 1). This suggests that development rate is similar between thermal environments. *T. molitor* developmental time is altered by temperature, diet, humidity, population density, parental age, light regime, and oxygen concentration (Connat et al., 1991; Greenberg and Amos, 1996; Ludwig, 1956; Murray, 1968; Tyshchenko and Ba, 1986; Urs and Hopkins, 1973; Weaver and Macfarlane, 1990). The duration of the complete life-cycle was comparable with data reported for *T. molitor* standarizing by the different rearing temperatures (see Damborsky et al., 1999; Gerber 1975). In general, development time is reduced when insects are exposed to fluctuating temperatures (see Hagstrum and Leach, 1973; Lamb and Gerber, 1985; Liu et al., 1995). The previous was only found in adult stage, where beetles of a variable environment have a significantly shorter life period than beetles from a constant thermal environment probably due to cellular damage (e.g. ice formation) and/or energetic cost of activation of the physiological and biochemical machinery necessary to face extreme events during the larval stage (e.g. *Hsp*70 gene expression see Dong et al., 2008; Hofmann and Somero, 1995). The reduction of adult time in the variable environment could have fitness consequences, because tenebrionids are sexually mature about 7–12 days after metamorphosis and the ovoposition begins 7 days as minimum giving a total of 14 days for one batch of eggs (Menon and Nair, 1972), which is almost the life period of an adult in variable thermal environment (15.9 ± 9.8 days). In many ectotherms, temperature conditions experienced during development may affect the final adult (Atkinson, 1994; Atkinson and Sibly, 1997). Ectothermic animals exhibit two different kinds of plasticity in response to temperature: thermal performance curves, in which an individual's performance varies in response to current temperature; and developmental reaction norms, in which the trait value of a genotype varies in response to developmental temperatures experienced over some time period during development (Kingsolver et al., 2004). Our results support the idea that *T. molitor* has a developmental reaction norm response because several traits of beetles are not affected in their rates during larval development but they have consequences in the adult stage (see below).

4.2. Thermal performance traits

Temperature thresholds vary among insect life-stages (Bowler and Terblanche, 2008; Giebink et al., 1985; Sanborn et al., 1982), and within a life-stage among different instars (Chown, 2001; Hutchison et al., 1986). This suggests that temperature thresholds are dynamic and change with insect age (Bowler and Terblanche, 2008; Howe, 1967). Our results indicate that larvae acclimated in a variable thermal environment are more cold-tolerant than larvae of a constant environment. Nevertheless, these results are reverted in adult stage, where individuals in a constant environment tolerate lower temperatures than adult in a variable environment (see Fig. 2). The previous demonstrates that the change of ontogenetic stage and thermal environment modifies thermotolerance of *T. molitor*. Boina and Subramanyam (2004) in *Tribolium confusum* have shown the same pattern with heat tolerance. A more complete explanation for this change in cold tolerance is that this trait can be switched on or off as a consequence of, or as a response to, environmental challenge (Spicer and Gaston, 1999). In other words, an early stimulus or environmental challenge that operates in a critical or sensitive period (larval phase) can result in a long-term change in the structure or traits of an organism (Lucas, 1991). This phase may exist because there are physiological limitations on how rapidly an organism can respond developmentally to environmental challenges (Schlichting and Pigliucci, 1998). Nevertheless, independent of the thermal environment to which individuals belong, the more cold-tolerant stage was the larvae, showing evidence of a higher conservation of thermal tolerance traits compared to adults (Mahroof et al., 2005). In comparison to adults *T. molitor* larvae have less mobility and are more exposed to temperature fluctuation because adults can quickly thermoregulate behaviorally (for an example see Pearson and Lederhouse, 1987). The better performance of larvae to cold shock could be explained as an adaptation to these sensible stage conditions. On the other hand, a clear trade-off in cold tolerance is found in a variable thermal environment between larval and adult stages. This outcome suggests that the chill-coma of late instars is affected by thermal conditions in early instars. These intra-individual trade-offs between cold tolerance (higher in the variable environment) during larval phase and chill-coma in adult stage (lower in the constant environment) could be interpreted as a change in the organism allocation strategy. Furthermore, larvae of the variable thermal environment showed a trade-off between cold tolerance and recovery time indicating that biochemical and physiological functions to face extreme events are energetically costly (see also Chown and Nicholson, 2004). Interestingly, our study does not support one of the explanations for persistence of complex life-cycle that sets the uncoupling of phases in the development of an individual as an adaptive mechanism to respond to environment in each ontogenetic stage (Moran, 1994; Wilbur, 1980). In contrast, it seems that the variable environment experienced during early ontogenic stages of beetles have large effects during their adult phase.

The physiological basis governing the recovery time is quite complex (David et al., 2003) but it is believed that the increase in cold recovery, previously reported in adults, could be product of accumulated injuries by the ice formation in nerves during early ontogeny (Castañeda et al., 2005; Macdonald et al., 2004) which imply a decrease in the biological function late in ontogeny (Sibley and Atkinson, 1994). At low temperatures, internal ice formation produces an ionic disequilibrium, depolarizing or hyperpolarizing the nerve cells which generate a loss of excitability and then motionless (Hosler et al., 2000; Sinclair et al., 2004). Previous arguments, and the periodicity of thermal extreme events in a variable environment, can explain that the recovery time of beetles (larvae and adults) in this environment delays by about 3 min the time necessary to regain an upright position than the beetles of constant thermal environment. Recovery time and chill-coma are both estimators of cold tolerance in terrestrial arthropods, and appear to be very informative and rapid indices for describing thermal adaptation (Castañeda et al., 2005; David et al., 2003; Hoffmann et al., 2002). This tolerance probably enhances over-winter survival and provides a fitness advantage during the cold season (Gibert et al., 2001).

4.3. Hsp70-like gene expression

It has been reported that chaperone proteins are expressed and act both in cold and heat events (Feder and Hofmann, 1999; Tine et al., 2010). At low temperatures the synthesis and expression of *Hsp70* occurs after the point of freezing, because the depressed organism's metabolism is such that it is impossible to activate the physiological machinery necessary to begin to synthesize the chaperone proteins, which occurs primarily in the recovery time, therefore providing thermal tolerance for subsequent extreme events (Krebs and Bettencourt, 1999; Chown and Nicholson, 2004). The difference in *Hsp70*-like gene expression between variable and constant environments (larvae) can be explained by the fluctuations in temperature that more than once, presented extreme events that triggered the physiological machinery

(from −2.5 to 43 °C). Probably, a variable environment forced the system to continuously respond with a higher expression of the gene giving greater tolerance to these individuals. Gene expression pattern was well correlated with the variable environments, variable environments trigger higher gene expressions, which was independent of the developmental stage. Then, the better performance of larvae reared on variable environments under a temperature shock could be correlated with a higher *Hsp70*-like gene expression. Nevertheless, although the expression of this gene is also higher (and even higher than in larvae) in adults of the variable environments, these individuals have a poor performance under temperature shocks. The last could be explained in different ways. During larval stage the thermotolerance could be achieve by redundant methods (e.g. through antifreeze proteins; see Strom et al., 2005) and therefore it is possible that many genes could be involved in this process. In fact, several species have shown the presence of more than one heat-inducible *Hsp70* gene in the same organism (Artavanis-Tsakonas et al., 1979; Lindquist, 1986; Ueda and Boettcher, 2009) that can be switched in the life-cycle of an individual. We cannot discard that other members of the Hsp gene family could be acting in the observed responses, and future experiments should analyze a broad spectrum of gene expression under temperature variable environments.

Even the expression of *Hsp70*-like gene is high in adults of the variable environment; this is not well correlated with the performance of these individuals under a cold temperature shock. As was discussed early, the variable environment experienced during larval stage has effects on the adult stage performance. Then, it possible to argue that even the expression of the gene is high, the molecular machinery that responds to this protein could be damaged or there is not enough energy to trigger an efficient response. When the chaperon protein and gene expression pattern was analyzed in another tenebrionid (*Tribolium castaneum*), gene expression was down regulated in old larvae and was expressed to high levels in adult stage (Mahroof et al., 2005). The expression of *Hsp70* has also been shown to vary during development in several species in different directions (Chang, 2005; Dix, 1997; Feder and Hofmann, 1999; Giudice et al., 1999; Karouna-Renier et al., 2003; Mahroof et al., 2005; Ueda and Boettcher, 2009; Wood et al., 1999; Yeh and Hsu, 2002). Moreover, it has been shown in a migratory locust that phenotypic variation of thermotolerance (i.e. *Hsp*70) is heritable and heritable characteristics differed among different stages of locust egg development and environments (Wang and Kang, 2005).

5. Conclusions

We have demonstrated that chill-coma, recovery time and *Hsp70* gene expression are plastic in response to thermal environment, but also that these traits change significantly depending the ontogenetic stage, implying that the response of adult individuals is linked to early stages of the life-cycle. Therefore, trait trade-offs and development constraints are important factors to consider in estimations of thermal performance in ectotherms. Furthermore, the responses of thermal physiological traits in adults, as chill-coma and recovery time, are not linked with chaperone protein gene expression, which make aware generalizations about thermotolerance of organism only evaluating proteins of heat shock. We show that thermal environment during early ontogeny conditioned response to cold in adult stage but also in the same ontogenetic stage highlighting the importance to consider the entire life-cycle (mainly susceptible early stages) and several traits to predict consequences of increase in temperature variance in the actual scenario of climatic change.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jtherbio.2011.06.011.

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