doi: 10.1111/j.1420-9101.2008.01630.x

HSP70 expression in the Copper butterfly *Lycaena tityrus* across altitudes and temperatures

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Keywords:

acclimation;
clinal variation;
developmental plasticity;
ELISA;
global warming;
stress resistance;
thermal adaptation;
thermal stress.

Abstract

The ability to express heat-shock proteins (HSP) under thermal stress is an essential mechanism for ectotherms to cope with unfavourable conditions. In this study, we investigate if Copper butterflies originating from different altitudes and/or being exposed to different rearing and induction temperatures show differences in HSP70 expression. HSP70 expression increased substantially at the higher rearing temperature in low-altitude butterflies, which might represent an adaptation to occasionally occurring heat spells. On the other hand, high-altitude butterflies showed much less plasticity in response to rearing temperatures, and overall seem to rely more on genetically fixed thermal stress resistance. Whether the latter indicates a higher vulnerability of high-altitude populations to global warming needs further investigation. HSP70 expression increased with both colder and warmer induction temperatures.

Introduction

Variation in environmental conditions is a significant source of mortality in nature (Willmer *et al.*, 2000), and in this context temperature is thought to be a key factor and consequently considered an important selective agent (Clarke, 2003; Hoffmann *et al.*, 2003). Most organisms experience variable thermal environments, posing substantial challenges for key elements of fitness such as survival and reproduction (Dahlhoff & Rank, 2007). Consequently, the evolution of behavioural, physiological and molecular mechanisms to cope with stressful conditions is expected and generally found (Hoffmann *et al.*, 2003; Sørensen *et al.*, 2003). Facing rapidly changing climatic conditions at the global scale (e.g. Parmesan *et al.*, 1999; Hitch & Leberg, 2007), organisms will have to adapt to the changing environment to avoid extinction (Angilletta *et al.*, 2002; Helmuth, 2002; Dahlhoff & Rank, 2007). However, the ability to cope with temperature extremes rather than different mean temperatures is probably of much greater relevance for species survival and thermal adaptation (Anderson *et al.*, 2003).

One well-known mechanism to cope with extreme temperatures is the expression of stress-inducible heat-shock proteins (HSPs), which are thought to play an important ecological and evolutionary role in thermal adaptation (Sørensen *et al.*, 2003). Most HSPs function as molecular chaperones participating in protein folding and unfolding, and they are essential in the cell's response to a variety of damaging conditions (Parsell & Lindquist, 1994). However, with respect to terrestrial arthropods, our knowledge on the role of HSPs is almost entirely restricted to a few model organisms (mainly *Drosophila*; Krebs and Loeschcke, 1994a, b; Lansing *et al.*, 2000; Krebs & Holbrook, 2001; Sørensen *et al.*, 2005). Studies using such model organisms have yielded some insight into the complex relationships between temperature stress resistance and particularly HSP70 expression (e.g. Sørensen *et al.*, 2005; Dahlhoff & Rank, 2007). However, how much of this applies to terrestrial arthropods in general is uncertain and studies on nonmodel organisms are needed to fill this gap.

Although the upregulation of stress-inducible HSPs may help organisms to cope with stress thus enhancing survival (Sørensen *et al.*, 2003; Dahlhoff, 2004; Dahlhoff & Rank, 2007), this may involve substantial costs. HSP expression

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consumes much cellular energy and competes with the housekeeping metabolism, causing reduced cell growth rates and a reduction in productivity (Krebs and Loeschcke, 1994a, b; Krebs & Holbrook, 2001; Robertson, 2004). Consequently, HSP induction may increase vulnerability to other stresses (Feder & Hofmann, 1999; Morales *et al.*, 2006). Continuous or frequent exposure to stress may therefore reduce the expression of HSP70 through evolution, as the associated costs may outweigh its benefits (Sørensen *et al.*, 1999, 2001; Lansing *et al.*, 2000). Such variation in the expression of HSPs may limit the distribution and abundance of organisms along steep ecological (e.g. thermal) gradients in nature (Roberts *et al.*, 1997; Dahlhoff *et al.*, 2001; Dahlhoff, 2004; Hofmann, 2005). If genetic variation in physiological responses is found over short geographical distances, such as altitudes, this would strongly support the notion of adaptive evolution via directional selection (Dahlgaard *et al.*, 2001).

Here, we transfer *Drosophila* expertise (expression of stress-inducible HSP) to a nonmodel organism, the temperate-zone butterfly *Lycaena tityrus*. We compare expression patterns across replicated populations originating from different altitudes, and at the same time across different ambient temperatures. We specifically addressed the following questions. (1) Does the expression of HSP70 vary across populations from different altitudes? In high-altitude populations, being exposed to harsher environmental conditions in their natural environment, a lower level of HSP expression might be expected at any given stress level, as the stress is experienced as less of an exceptional situation. (2) Does HSP70 expression vary across developmental/acclimation and induction temperatures with increased HSP expression at both ends of the temperature scale?

Materials and methods

Study organism and butterfly rearing

Lycaena tityrus (Poda, 1761) is a widespread temperate-zone butterfly, ranging from western Europe to central Asia (Ebert & Rennwald, 1991). The species is bivoltine with two discrete generations per year in most parts of its range, although populations with one or three generations per year occur (Ebert & Rennwald, 1991; Tolman & Lewington, 1998). The principal larval host-plant is *Rumex acetosa* L., but some congeneric plant species, such as *R. acetosella* L. and *R. scutatus* L., are utilized as well (Ebert & Rennwald, 1991; Tolman & Lewington, 1998). Mated females from replicated low- [Rhineland-Palatinate, Germany: 250 a.s.l., (50°30′N, 7°58′E; $N = 13$, population 1); Bavaria, Germany: 600 a.s.l. (47°42′N, 11°24′E; $N = 6$, population 2)] and high-altitude [South Tyrol, Italy: 2010 a.s.l. (46°43′N, 10°52′E; $N = 23$, population 3); Tyrol, Austria: 2050 a.s.l. (46°52′N, 11°01′E; $N = 21$, population 4)] populations were caught in July/August 2007 in the field and transferred to Bayreuth University.

For egg laying, butterflies were kept in a climate chamber at 27 °C, high humidity (~70%) and a photoperiod of light 18 h : dark 6 h. Females were placed group-wise, separated by population, into translucent plastic boxes (15 L) and provided with *R. acetosa* (oviposition substrate), fresh flowers (*Crepis* sp., *Achillea millefolium*, *Bistorta officinalis*, *Leucanthemum vulgare*) and a highly concentrated sucrose solution (for adult feeding). Eggs were collected daily, pooled within populations, and transferred to small glass vials. After hatching, larvae were randomly divided into two rearing temperatures (20 and 27 °C; L18 : D6 and 70% relative humidity throughout). Larvae were first reared in groups of 10 individuals, but during the last two larval stages individually in translucent plastic boxes (125 mL), containing moistened filter paper and fresh cuttings of *R. acetosa* in ample supply. Boxes were checked daily and supplied with new food when necessary. Following adult eclosion, butterflies were separated by eclosion day and population and transferred to cylindrical hanging cages kept at their respective rearing temperature. They were provided with fresh flowers (*Crepis* sp., *A. millefolium*, *Polygonum bistorta*, *L. vulgare*) and a highly concentrated sucrose solution for adult feeding.

Experimental design and sample preparation

On day 2 after eclosion, the butterflies from both rearing temperatures and each population were randomly divided among five treatments, being either exposed for 1 h to 1, 10, 20, 27 or 37 °C. We observed no mortality induced by these treatments. Thereafter, butterflies were back-transferred to their respective rearing temperature for 1 h to allow for the possible upregulation of HSP, after which they were frozen at −78 °C for later analysis. In total, 560 individuals in 80 groups (two altitudes × two replicates × two rearing temperatures × five test temperatures × two sexes) were exposed to the different temperature treatments. Samples were prepared for measuring HSP expression by removing heads, legs, wings and abdomen. Thorax fresh mass was determined to the nearest 0.01 mg (Sartorius microscale MC 210 P, Sartorius AG, Goettingen, Germany). Thereafter, thoraxes were homogenized in 400 μL of ice-cold phosphate-buffered saline (PBS), containing 200 mM PEFAbloc and a 1 vol% antiprotease cocktail (100 μL/mL pepstatin A, 50 μL/mL leupeptin, 10 mM benzamidine, 10 mM sodium metabisulfite), and then centrifuged for 30 min at 13 000 rpm (11000 *g*) at 4 °C. The supernatant was divided into three replicate samples of 100 μL each (to ensure extra samples if necessary), transferred to 0.5-mL Eppendorf tubes, and frozen again at −78 °C.

ELISA

For *L. tityrus*, HSP70 expression patterns were never investigated before and consequently no specific anti-

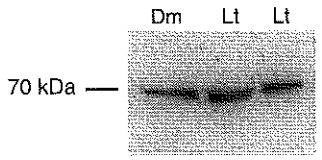

Fig. 1 Western blot showing bands of similar sizes around 70 kDa in *Drosophila melanogaster* (Dm) and *Lycaena tityrus* (Lt). The antibody recognized Hsp70 in *L. tityrus* with comparable affinity as in *D. melanogaster*.

bodies were known. Western blotting was used to confirm measuring a protein of the predicted size (70 kDa) for the antibody used (Fig. 1). HSP70 expression was measured by an enzyme-linked immunosorbent assay (ELISA) following the protocol of Dahlgaard *et al.* (1998) with some modifications, i.e. by using an HSP70-specific monoclonal antibody (Clone 5A5, mouse anti-rabbit, 1 : 750, Abcam, UK) and an HRP-conjugated secondary antibody (Polyclonal Rabbit Anti-Mouse IgG; DAKO A/S, Glostrup, Denmark). The primary antibody detects both, the constitutive and induced HSP70 family members (referred to as HSP70 for simplicity here). Linearity was verified by testing signal responses with increasing HSP70 concentration ($r = 0.894$). On each plate, all 80 groups (40 groups × two sexes) were represented, and all samples were measured on four replicate plates. The resulting signal was measured by a spectrophotometric microplate reader (EL_x 800; Bio-Tek Instruments, Bad Friedrichshall, Germany) at 562 nm. To standardize between plates, all data were adjusted to plate mean values. HSP70 expression is given as mean value of the four replicate plates relative to standardized protein content of 30 μL/mL [by means of BCA assays (Pierce Biochemicals, Rockford, IL, USA) according to the manufacturer's instructions]. With the same method total protein content of thoraxes was determined.

Statistical analyses

HSP70 expression was analysed using nested analyses of co-variance (ANCOVAS), with altitude, rearing temperature, induction temperature and sex as fixed effects and replicate population (nested within altitude) as random effect. Thorax mass and total protein content (in % of thorax mass) were added as covariates. Minimum adequate models were constructed by removing nonsignificant interaction terms. Pairwise comparisons were performed employing Tukey's HSD. All statistical tests were performed by using JMP (4.0.0) or STATISTICA (6.1). Unless otherwise stated, least square means ± 1 SE are given in the text.

Results

All main factors (except altitude) as well as three two-way interactions significantly affected HSP70 expression

Table 1 Nested ANCOVAS for the effects of altitude, replicate population (nested within altitude), rearing temperature, induction temperature and sex on HSP70 expression.

	MS	d.f.	*F*	*P*
Altitude	0.08	1, 2	0.5	0.5499
Replicate (altitude)	0.16	2, 545	37.9	**< 0.0001**
Rearing temperature	0.14	1, 545	33.6	**< 0.0001**
Induction temperature	0.06	4, 545	15.4	**< 0.0001**
Sex	0.02	1, 545	6.4	**0.0116**
Altitude × rearing temperature	0.05	1, 545	11.2	**0.0009**
Altitude × sex	0.12	1, 545	27.3	**< 0.0001**
Rearing temperature × sex	0.04	1, 545	9.6	**0.0020**
Thorax mass	0.001	1, 545	0.3	0.5904
Protein content	0.002	1, 545	0.4	0.5248
Error	0.004	545		

Thorax mass and thorax protein content (covariates) were added as covariates. Minimum adequate models were constructed by removing nonsignificant interaction terms. Significant *P*-values are given in bold.

in *L. tityrus* (Table 1). A significant interaction between altitude and rearing temperature indicates that low-altitude animals showed a strongly increased HSP70 expression at the higher compared with at the lower rearing temperature, with a comparable response being absent in high-altitude animals (Figs 2 and 3). Further, the increase in HSP70 expression at the higher rearing temperature was much more pronounced in females than in males, with the latter showing even a slight decrease in two of four populations (significant rearing temperature × sex interaction; Figs 2 and 3). Females showed much higher levels of HSP70 expression compared with males in low-altitude populations, whereas sex differences were almost absent in high-altitude populations (significant altitude × sex interaction; Figs 2 and 3).

As indicated by the significant main effects (Table 1), HSP70 expression was overall higher for individuals

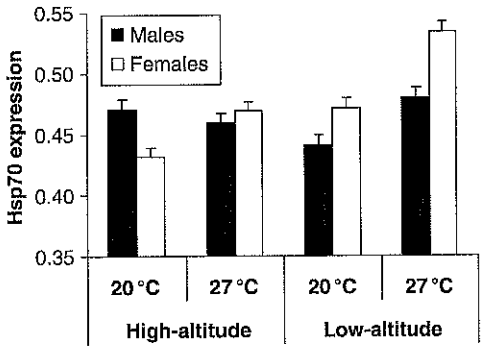

Fig. 2 Means (+1 SE) of HSP70 expression level for *Lycaena tityrus* males (black bars) and females (open bars) across different rearing temperatures (20 and 27 °C) and altitudes. Data were pooled across two replicate populations and five induction temperatures.

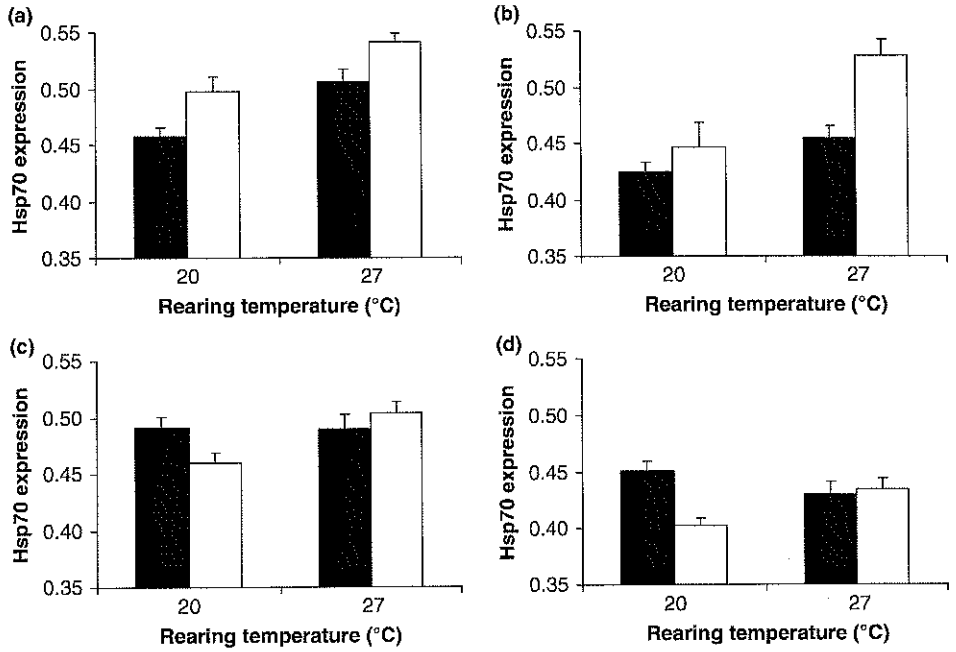

Fig. 3 Mean (+1 SE) of HSP70 expression level for *Lycaena tityrus* males (black bars) and females (open bars) across different rearing temperatures (20 and 27 °C) for populations 1 (a), 2 (b), 3 (c) and 4 (d). Populations 1 and 2 are from low altitudes, whereas populations 3 and 4 are from high altitudes. Data were pooled across induction temperatures.

reared at 27 °C (0.486 ± 0.005) compared with those reared at 20 °C (0.454 ± 0.004; caused by the large effects of rearing temperature on low-altitude animals and a lack of response in high-altitude animals), and overall higher in females (0.477 ± 0.005) compared with in males (0.463 ± 0.004; owing to pronounced sex differences in low-altitude and a lack thereof in high-altitude populations). HSP70 expression was further the highest at an induction temperature of 1 °C and the lowest at 20 °C (1 °C: 0.504 ± 0.009 ≥ 37 °C: 0.484 ± 0.010 ≥ 10 °C: 0.467 ± 0.006 > 27 °C: 0.448 ± 0.006 = 20 °C: 0.446 ± 0.005; Tukey HSD after ANCOVA; Fig. 4). Neither covariate significantly affected HSP70 expression.

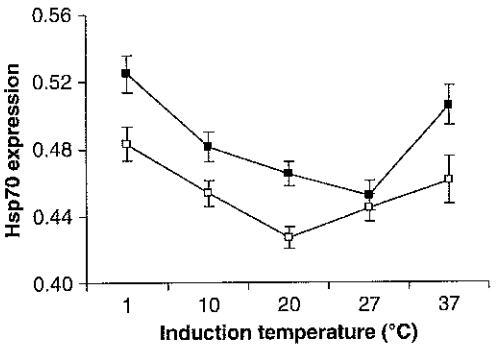

Fig. 4 Means (±1 SE) of HSP70 expression level for *Lycaena tityrus* across rearing temperatures (20 °C: white symbols; 27 °C: black symbols) and induction temperatures.

Discussion

Facing rapid human-induced climate change, understanding the mechanisms by which organisms respond to environmental variation received increasing attention (Dahlhoff & Rank, 2007). In this context, interest in the molecular and physiological functions of HSP increased over recent years (Parsell & Lindquist, 1993; Sørensen *et al.*, 2003). However, very low amount of data is presently available for nonmodel organisms such as the butterfly species studied here. Such data, however, are needed for assessing the generality of findings on the role of the heat-shock response for thermal adaptation obtained from model species.

The ability to express HSPs under thermal and other stresses is an essential mechanism to cope with unfavourable conditions for ectothermic organisms (Sørensen *et al.*, 2003; Dahlhoff, 2004; Dahlhoff & Rank, 2007). Although differentiation in HSP expression across altitudinal and latitudinal clines can be expected (Garbuz *et al.*, 2003; Sørensen *et al.*, 2005), there were no overall effects of altitude in *L. tityrus* detectable (but see below for interactive effects). Consequently, high-altitude butterflies did not show generally lower levels of HSP expression. However, whereas high-altitude butterflies responded only marginally to differences in rearing

temperature, HSP expression increased substantially at the higher temperature in low-altitude butterflies. Note in this context that 27 °C is a relatively high rearing temperature for a temperate-zone butterfly, especially for high-altitude animals, and that such conditions may amplify otherwise obscured phenotypic differences between populations (Hoffmann & Parsons, 1991; Hoffmann & Merilä, 1999; Blanckenhorn & Heyland, 2004). Nevertheless, the high-altitude environment is certainly generally cooler and probably also less predictable than the low-altitude environment (Franz, 1979), causing potentially higher levels of stress. Thus, we assume that the reduced plasticity in high-altitude relative to low-altitude animals in response to long-term exposure to 27 °C might be related to the fact that elevated heat stress is less exceptional in high-altitude settings (cf. Sørensen *et al.*, 1999, 2001; Lansing *et al.*, 2000).

Under such conditions, the heat-shock response would consume a large amount of cellular energy, which may cause an energy debt (Gething & Sambrook, 1992; Krebs & Loeschcke, 1994a, b; Dahlhoff & Rank, 2007). Such energy losses may favour alternative mechanisms to cope with high-altitude conditions. In *L. tityrus*, one mechanism seems to be allelic variation at the PGI locus, with a particular genotype, being associated with increased cold stress resistance, dominating in high-altitude populations (I. Karl, T. Schmitt & K. Fischer, unpublished data). Thus, these populations might rely in the first place on genetically fixed resistance helping to conserve energy under the harsher environmental conditions. Whether concomitant reductions in flexible responses confer a relatively higher vulnerability to global warming needs further investigation. The pronounced plastic response in low-altitude populations, by contrast, might be related to occasionally occurring fast increases in daily temperatures, warranting a molecular system which is able to respond rapidly (Dahlgaard & Loeschcke, 1997).

Overall, environmental effects on HSP70 expression were more pronounced than differences across low- and high-altitude populations, although rearing temperature effects were largely restricted to low-altitude animals and females. Moreover, butterflies clearly responded to the different induction temperatures used. Although often only high temperatures are used for HSP induction (e.g. Dahlgaard *et al.*, 1998; Sørensen *et al.*, 2001, 2005), low temperatures are also known to upregulate HSPs (Yocum, 2001; Hoffmann *et al.*, 2003; Michaud & Denlinger, 2005). Accordingly, in *L. tityrus* the expression of HSP70 increased both towards high and low temperatures. Most interestingly, the lowest expression levels were found at the same temperature the respective individuals were reared at (i.e. for individuals reared at 27 °C at an induction temperature of 27 °C; and for individuals reared at 20 °C at 20 °C). This may indicate that a change in the thermal environment generally induces some stress, supporting the beneficial acclimation hypothesis (Huey *et al.*, 1999; Woods & Harrison, 2002). Note though that this pattern was not statistically supported by a significant interaction between rearing and induction temperature.

In addition to the patterns discussed above, females from low-altitudes or females reared at the higher temperature showed higher expression levels than males. Sex differences in HSP expression are commonly found in studies of *Drosophila*, (Dahlgaard *et al.*, 1998; Sørensen *et al.*, 2005). In our study, if anything, females rather than males showed increased expression levels. Note though that HSP70 expression was measured 1 h after induction, and thus we have only data for one time point. This may have some implications for the patterns found, as in *Drosophila* males the increase in HSP70 expression is less steep than that for females, and the highest values were found around 2 h after hardening (Dahlgaard *et al.*, 1998; Sørensen *et al.*, 2001). Nevertheless, as sexes also varied across rearing temperatures with females showing increased HSP70 expression (accompanied by an increased heat resistance; Karl *et al.*, 2008a), there might be a true difference in the heat-shock response across sexes in *L. tityrus*. As for example in *Drosophila ananassae* heat stress strongly affected survival and additionally reduced female fecundity (Sisodia & Singh, 2006). Such differences in HSP expression may reflect the females' higher need for protection against thermal stress.

Conclusions

As most of our knowledge on patterns of HSP expression stems from studies using *Drosophila* as a model organism, we here investigate population and environmental effects on HSP70 expression in the Copper butterfly *L. tityrus*. Environmental effects according to different rearing and induction temperatures were, overall, more pronounced than differences across populations. The latter effects were largely restricted to low-altitude butterflies reared at the higher temperature. These and other results (Karl *et al.*, 2008b) may suggest that high-altitude butterflies may rely more on genetically fixed resistance to environmental variation than lowland ones, whereas low-altitude animals showed higher levels of plasticity. HSP70 expression increased with increasingly colder and warmer induction temperatures. The latter finding gave some support for the beneficial acclimation hypothesis, as the lowest levels of HSP expression coincided with the respective rearing temperature. This is the first study on HSP70 expression in a Copper butterfly, laying the fundament for future investigations within a comparative context.

Acknowledgments

We thank I. Thamke for help with butterfly rearing, and two anonymous reviewers for constructive criticism. Financial support was provided by the German Research

Council (DFG grants no. Fi 846/1-3 and 1-4 to KF), the European Science Foundation (ESF) within the research networking program 'Thermal adaptation in ectotherms' (LESC EG/1424), and the International School of Biodiversity Sciences (ISOBIS), University of Aarhus, Denmark.

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Received 11 April 2008; accepted 4 September 2008