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Expression of Stress Proteins of HSP70 Family in Response to Cold in *Myrmica* Ants from Various Geographic Populations

A. V. Maisov[a]*, Yu. I. Podlipaeva[b], and V. E. Kipyatkov[a]

[a] *Department of Entomology of St. Petersburg State University*
[b] *Institute of Cytology of RAS, St. Petersburg, Russia*
**e-mail: owlsmay@mail.ru*

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Abstract—Expression of HSP70 is induced by stress factors, including sublethal chilling. However, the role of HSP70 for overcoming the consequences of cold stress is not clear. If it is positive, the level of HSP70 expression might be higher in populations from cold climates. Using the immunoblotting technique we investigated dynamics of HSP70 expression in response to cold stress in two *Myrmica* species (*M. rubra* and *M. ruginodis*) from three localities of different latitudes (50, 60 and 67°N). The results showed that in the more thermophilic species *Myrmica rubra*, expression of HSP70 after cold stress was higher. Within both species, HSP70 expression did not show a direct relationship with latitude. The southernmost population of *M. rubra* and northernmost population of *M. ruginodis* displayed the fastest and the most intense response. However, two other populations of *M. rubra* were similar in timing and intensity of the response, while in *M. ruginodis* the intermediate population showed the slowest and weakest response. The data suggest that expression of HSP70 may play essential role for adaptation to cold only in the northernmost population of *M. ruginodis*

Key words: *HSP70, stress, cold tolerance, geographic variation, Myrmica.*
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Living organisms frequently face the problem of survival in stress conditions when normal activity is impossible. At the cellular level, stress induces biochemical damages that reduce the functionality of tissues and organs, resulting in a decrease in competitive ability and even the death of an organism. Among the lines of molecular defense against such damages are heat shock proteins (HSPs), whose expression is induced by various stress factors, and which help the cell to manage the accumulation of denaturated proteins (Feder and Hofmann, 1999, Sørensen et al., 2003).

Although the molecular role of HSPs is well understood, their role as protectors from natural stresses should be verified (Feder and Hofmann, 1999). Studies of their involvement in stress protection in ants are scarce; the only such example concerns protection from heat stress in desert species of *Cataglyphis* (Gehring, Wehner, 1995). In temperate and boreal zones, cold stress is much more likely, but whether HSPs are implicated in protection from cold is not clear, even for traditional model organisms (*Drosophila*) (Hoffmann et al., 2003). However, pronounced expression of HSPs after short sublethal chilling (Burton et al., 1988, Joplin et al., 1990, Denlinger et al., 1991) may indicate their potential role for cellular reparative functions.

Under laboratory conditions *Myrmica* ants tolerate 0°C for 24 h with no mortality, but a high percent of them die in 7 days at 5°C; i.e., these temperatures are stressful for them. In the active period of life, *Myrmica* colonies experience similar stress in spring after hibernation when they attempt to settle in microhabitats above the soil surface, where warmth accumulates during the daytime (Brian, Brian, 1951, Brian, 1972) but chilling occurs at night. The severity of regular night chilling increases with the latitude, and so do the frequency and duration of unexpected cold events. Thus, *Myrmica* might have an advantage from HSP synthesis, resulting in genetically inherited elevated levels at higher latitudes.

We aimed to investigate whether HSPs give the above-mentioned advantage for ants. For this purpose we compared the expression of HSP70 in two *Myrmica* species on colonies collected from three localities of different latitudes. Such an approach, involving a natural climatic gradient, allows one to elucidate the proteins' role in adaptation to cold stress under natural conditions (Feder and Hofmann, 1999).

MATERIALS AND METHODS

Species and populations. We studied two common palearctic species, *M. rubra* and *M. ruginodis*, collected near the following three geographic points: Borisovka (50°36′ N, 36°01′ E), Peterhof (59°53′ N, 29°52′ E), and Luvenga (67°09′ N, 32°24′ E). Colonies

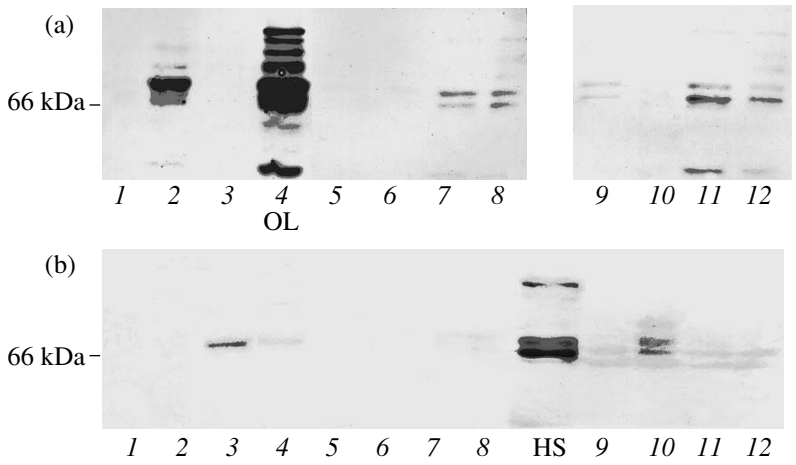

Fig. 1. Dynamics of HSP70 expression in response to short cold stress in *M. rubra* (a) and *M. ruginodis* (b) from three areas of various latitudes: from Borisovka (50° N, *lane 1–lane 4*), Peterhof (60° N, *lane 5–lane 8* and HS) and Luvenga (67° N, *lane 9–lane 12*). For each locality, the numbered lanes correspond left to right to control (no stress) and timings 0, 3, 6 h after cold stress (1 h at 3°C). HS, response to heat stress (1 h at 37°C) after 3 h (only for *M. ruginodis*). OL, error (overload) at the start of electrophoresis. Position of 66 kDa molecular weight marker is shown on the left.

were collected in a forest, except for *M. rubra* from Luvenga where the species inhabits only open areas. Expression of HSP70 was measured twice, in 2004 and 2005, each year on newly collected colonies.

Collection and maintenance. From each natural colony, a numerous fraction (250–300 worker ants, 1–2 queens, larvae, and pupae) was collected with an exhauster into plastic vials lined with a moistened sponge and then transported to the laboratory. Fractions were settled in closed plexiglas formicaria (Kipyatkov et al., 2005), whose inhabitable section provided the shading and high relative humidity that is normal for the inner environment of natural ant nests. Before experiments, the insects were kept at 20°C with a daily photoperiod of 22 h of light and 2 h of dark. We fed the ants with pieces of moistened raisins and laboratory insects, including cockroaches *Nauphoeta cinerea* and flies *Calliphora vicina.* Food was renewed every third day.

Experimental procedure. The expression of HSP70 in response to cold was assayed in one colony from each locality after no less than 1.5 months of laboratory maintenance. In 2004, we placed two random samples of 50 worker ants from each colony into containers with gauze cover and moistened sponge and subjected one of the samples to cold stress (3°C, 1h) while keeping control sample at room temperature. After a short recovery (20°C, 2 h), both samples were prepared for assay. In 2005, we took four samples from each colony, one for control and three experimental ones to determine the dynamics of the HSP amount after stress. The samples were prepared for assay after 0 h (simultaneously with control) and then after 3 and 6 h at 20°C. In 2005 we also assayed the expression of HSP70 after heat stress (1 h at 37°C and 3 h at 20°C) in *M. ruginodis* from the Peterhof population.

The separated head capsules of 40 insects from each sample were homogenized in 40 μl extraction buffer, 20 mM Tris-HCl, pH 7.5, 0.1 mM EDTA, 0.5 mM dithiothreitol (DTT) and 0.2 mM phenylmethanelsulfonilfluoride (PMSF). Prepared samples were kept at –20°C. After thawing, samples were centrifuged at 10 000 g for 15 min. Supernatant was used to determine the total protein concentration according to the routine technique (Lowry et al., 1951).

Sample proteins were separated by SDS-electrophoresis in 10% PAAG in a tris-glycine system (Laemmli, 1970). To prepare electrophoretic samples, supernatant was mixed with 4-fold Laemmli buffer (1% SDS, 5% β-mercaptoethanol, 10% glycerin) at a 3 : 1 ratio. Samples were incubated at 100°C in a water bath for 3–4 min. Electrophoresis was performed in 120 × 90 × 0.8 mm gel plates for 1.5 h at I = 10–12 mA and 2.5 h at I = 20–25 mA. Protein molecular weight was estimated with High Range Rainbow Molecular Weight Markers, 14–200 kDa (Amersham Biosciences, USA).

Bands of HSP70 were revealed by immunoblotting after a transfer of proteins to a nitrocellulose membrane (Towbin et al., 1979). In 2004, membranes were treated with anti-HSP70 rabbit polyclonal antibodies ROM1 obtained in Laboratory of Cell Defense Mechanisms, Institute of Cytology, RAS. In 2005, we used monoclonal anti-HSP70 antibodies SPA-822 (Stressgen technologies, Canada). Antibody binding with protein was detected with secondary biotin-labeled antibodies conjugated with alkaline phosphatase (Sigma, USA). Band staining was compared visually.

RESULTS

The amount of both constitutive and stress-induced HSP70 turned out to be higher in *M. rubra* than in *M. ruginodis* in all three populations (Fig. 1). The amount was particularly low in the Peterhof population of *M. ruginodis*: here we could not detect HSP70 in the first trial, when we used equal starting electrophoretic loads for both species (40 μg protein). To reveal HSP70 in all populations of this species, we had to increase the protein loading twice (80 μg). Response to heat in *M. ruginodis* (lane HS on the figure) was much more intense than to cold.

The dynamics of HSP70 expression observed in *M. rubra* (Fig. 1a) was as follows. Immediately after stress (0h), intensive HSP70 expression was registered in ants from the southernmost population (Borisovka, *lane 2*); in intermediate and northern populations (Peterhof and Luvenga), the HSP70 amount was a little lower than even in the control (*lanes 6* and *10*). After 3 h of recovery, the amount of HSP70 decreased in the southern population (*lane 3*) but increased in intermediate and northern populations (*lanes 7* and *11*). After 6 h, all populations displayed a band corresponding to a protein of 70 kDa molecular weight. Excessive lane staining due to unintended protein overloading prohibits a full comparison, but intermediate and northern populations display a similar intensity of 70 kDa bands. Expression of HSP70 in *M. rubra* assayed in 2004 with noncommercial polyclonal antibodies after 2 h of recovery showed similarities to those after 3 h in 2005. Control variants of both years were also similar.

In *M. ruginodis* (Fig. 1b) intense accumulation of HSP70 in the southern population (Borisovka) occurred after a 3-h recovery (*lane 3*). In the intermediate population (Peterhof), HSP70 band is hardly visible only after 6-h recovery (*lane 8*). In the northern population (Luvenga), HSP70 accumulation was registered immediately after cold stress (*lane 10*). Comparison of the results with those obtained in 2004 was problematic due to the insufficient protein loading used in that trial and the weak response of this species. However, as in 2005 (Kipyatkov et al., 2005b), the intensity of staining for constitutive HSP70 (control variants) was higher in the northern population, while the intensity of induced HSP70 bands (2 h after stress) was higher in the southern population.

DISCUSSION

The available literature gives two examples where the role of HSP70 was assessed in ecologically realistic conditions in natural populations living in a gradient of climate. Both examples concern the synthesis of HSP70 in response to heat stress in populations of the subtropical species *Drosophila buzzatii* inhabiting an altitudinal gradient (200–2300 m height) in the highlands of northwestern Argentina. The amount of heat-induced HSP70 was lower in populations from low-altitude areas with warmer climates (Sørensen et al., 2001, 2005). To explain this seemingly paradoxical situation, it was hypothesized that thermal adaptation of populations reduces stress caused by high temperature (to a certain limit) and, therefore, leads to less cell damage and less HSP70 expression.

Studies on the cosmopolitan species *Drosophila melanogaster* confirm this idea. Comparison of its standard strain and the strain originating from subequatorial Africa revealed a reverse relationship of the level of HSP70 expression and thermal tolerance (Zatsepina et al., 2001). Genetical analysis of strains from a latitudinal gradient along the eastern coast of Australia indicated the same pattern, as the frequency of HSP70-coding allele bearing an expression-suppressing insertion was higher in populations from warmer climates. Moreover, if natural selection for thermotolerance was simulated in the laboratory, the frequency increased up to fixation of such alleles in populations that were kept at high temperatures (Bettencourt et al., 2002). The findings indicate that the adaptation of populations to high temperatures is determined not by HSP70 expression, but by other mechanisms, whereas HSP70 apparently protects from short-term extremely high temperatures, but not from their long and frequent periods (Sørensen et al., 2003). Extreme heat stress in nature may be a rare event for adult *Drosophila*, which actively chooses its thermal environment (Feder et al., 2000). As the synthesis of HSP70 has some costs on the organismal and populational levels (slower growth and development, higher mortality), they may outweigh its benefits under mild stress and natural selection may then favor reduced HSP70 expression (Bettencourt et al., 2002, Sørensen et al., 2001, Zatsepina et al., 2001).

Less is known about relationship of HSP70 and cold stress. Although various factors elicit HSP response, some stress-specificity is believed to exist (Joplin et al., 1990, Denlinger et al., 1991, Sørensen et al., 2003). This point is confirmed by the different responses of *M. ruginodis* to heat and cold stresses in our study. Moreover, cold-induced HSP70 synthesis was usually observed only in post-stress period (Denlinger et al., 1991). The present results show that intensive synthesis of HSP70 in *Myrmica* may occur during cold treatment.

The main result of the present study is that, contrary to our expectations, we did not find a direct relationship between latitude and the intensity or timing of HSP70 expression. Geographic variation of the response showed by populations of *M. rubra* is rather in favor of the existing hypothesis on the reverse correlation between the stress response and climatic adaptation (Sørensen et al., 2005). Reformulated in terms of cold stress, the idea is that the colder the climate, the higher the adaptation is to cold in a population and the less stressful cold is. The reason for similarity of the stress response in *M. rubra* from Luvenga and Peterhof may be that in Luvenga, the species settles only in open habitats, probably compensating for an insulation defi-

ciency. Unlike *M. ruginodis*, distributed in the north up to the latitude of 70° (Vepsalainen et al., 1984), *M. rubra* distributes approximately to the Arctic circle (Elmes et al., 1999; Groden et al., 2005). Although in more southern parts of their range *M. rubra* colonies settle in forests, where they still prefer patches under openings in forest canopy, i.e., more favorable temperature conditions. It is also in agreement with the view that the amount of HSP70 is an indicator of stress level (equal chilling for both species is more stressful for the more thermophilic one).

It is more difficult to explain the response variation found among populations of *M. ruginodis.* The ecological preference of all the populations are uniform; they live in shaded habitats and, therefore, are more comparable. The pattern of reverse correlation between stress response and local climate holds here only in part, as the northernmost population (Luvenga) breaks it. It seems unlikely that the most rapid and intense response to 3°C of the Luvenga population indicates the most severe cold stress. It is likely that in this area, benefits from HSP70 expression outweigh the above-mentioned costs and selection favors it. This may be the case, for example, if unpredictable cold snaps are always harsh and temperatures around 3°C for the population is not only a stress factor, but also a signal of upcoming more severe cold. Of course, it is clear from publications on heat stress that the assumption of a direct stressor–HSP70 relationship in populations is much too speculative. Nevertheless, all investigators point to the positive role of HSP70 at extreme levels of stress that endanger the organism's life. To clarify the difference in HSP70 expression in response to cold among populations of *M. ruginodis*, we need more detailed studies of the microclimate in their habitats.

The investigation of HSP70 expression after cold stress in *Myrmica* ants allows us to make the following conclusions: 1. Interspecific variation indicates that the response mirrors the level of stress induced by low temperature. The more thermophilic a species is, the more stressful chilling is and the higher the HSP70 expression is that is observed. 2. Intraspecific variation also confirms the rule stated above, i.e., the warmer the climate inhabited by a population, the more severe stress is caused by chilling. It means that increased expression of HSP70 is not beneficial for adaptation to cold climate. 3. Exception from the rule is the northern population of *M. ruginodis*. Supposedly, it may benefit from expression of HSP70 due to a high probability of a lethal level (or duration) of low temperatures in the north. To verify this suggestion, we need a detailed study of the population's thermal environment.

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REFERENCES

1. Bettencourt, B.R., Kim, I.Y., Hoffmann, A.A., and Feder, M.E., Response to Natural and Laboratory Selection at the Drosophila HSP70 genes, *Evolution*, 2002, vol. 56, pp. 1796–1801.
2. Brian, M.V., Population Turnover in Wild Colonies of the Ant *Myrmica ruginodis, Ekologia Polska*, 1972, vol. 20, pp. 43–53.
3. Brian, M.V. and Brian, A.D., Insolation and Ant Population in the West of Scotland, *Trans. R. Entomol. Soc. Lond.*, 1951, vol. 102, pp. 303–330.
4. Burton, V., Mitchell, H.K., Young, P., and Petersen, N.S., Heat Shock Protection Against Cold Stress of *Drosophila melanogaster*, *Mol. Cell. Biol.*, 1988, vol. 8, pp. 3550–3552.
5. Denlinger, D.L., Joplin, K.H., Chen, C.-P., and Lee, R.E.,. Cold Shock and Heat Shock, in: *Insects at Low Temperature*, New York and London: Chapman and Hall, 1991, pp. 131–148.
6. Elmes, G.W., Wardlaw, J.C., Nielsen, M.G., Kipyatkov, V.E., Lopatina, E.B., Radchenko, A.D., and Barr, B., Site Latitude Influences on Respiration Rate, Fat Content, and the Ability of Worker Ants to Rear Larvae: a comparison of *Myrmica rubra (Hymenoptera: Formicidae)* Populations over Their European Range, *Eur. J. Entomol.*, 1999, vol. 96, pp. 117–124.
7. Feder, M.E. and Hofmann, G.E., Heat-shock Proteins, Molecular Chaperones and the Stress Response, *Ann. Rev. Physiol.*, 1999, vol. 61, pp. 243–282.
8. Feder, M.E., Roberts, S.P., and Bordelon, A.C., Molecular Thermal Telemetry of Free-ranging Adult *Drosophila melanogaster*, *Oecologia*, 2000, vol. 123, pp. 460–465.
9. Gehring, W.J. and Wehner, R., Heat Shock Protein Synthesis and Thermotolerance in Cataglyphis, an Ant from the Sahara Desert, *Proc. Natl Acad. Sci. USA*, 1995, vol. 92, pp. 2994–2998.
10. Groden, E., Drummond, F.A., Garnas, J., and Franceour A., Distribution of an Invasive Ant, *Myrmica rubra (Hymenoptera: Formicidae)* in Maine, *J. Econ. Entomol.*, 2005, vol. 98, pp. 1774–1784.
11. Hoffmann, A.A., Sørensen, J.G., and Loeschcke, V., Adaptation of Drosophila to Temperature Extremes: Bringing Together Quantitative and Molecular Approaches, *J. Therm. Biol.*, 2003, vol. 28, pp. 175–216.
12. Joplin, K.H., Yocum, G.D., and Denlinger, D.L., Cold Shock Elicits Expression of Heat Shock Proteins in the Flesh Fly, *Sarcophaga crassipalpis*, *J. Insect Physiol.*, 1990, vol. 36, pp. 825–834.
13. Kipyatkov, V.E., Lopatina, E.B., and Imamgaliev, A., Duration and Thermal Reaction Norms of Development Are Significantly Different in Winter and Summer Brood Pupae of the Ants *Myrmica rubra* Linnaeus, 1758 and

M. ruginodis Nylander, 1846 (Hymenoptera: Formicidae), *Myrmecologische Nachrichten*, 2005a, vol. 7, pp. 69–76.

14. Kipyatkov, V.E., Podlipaeva, Yu.I., and Maysov, A.V., First Demonstration of the Latitudinal Variation of Heat Shock Protein Content and Expression after Cold Shock in Ants *Myrmica rubra L.* and *M. ruginodis Nyl.*, in: *International Symposium on the Environmental Physiology of Ectotherms and Plants*, Roskilde, Denmark, 2005b, p. 41.
15. Laemmli, U.K., Cleavage of Structural Proteins during the Assembly of the Head of Bacteriophage T4, *Nature*, 1970, vol. 227, pp. 680–685.
16. Lowry, O.H., Rosenbrough, N.J., Farr, A.L., and Randall, R.F., Protein Measurement with the Folin Phenol Reagent, *J Biol. Chem.*, 1951, vol. 193, pp. 265–275.
17. Sørensen, J.G., Dahlgaard, J., and Loeschcke, V., Genetic Variation in Thermal Tolerance among Natural Populations of *Drosophila buzzatii*: Down Regulation of Hsp70 Expression and Variation in Heat Stress Resistance Traits, *Funct. Ecol.*, 2001, vol. 15, pp. 289–296.
18. Sørensen, J.G., Kristensen, T.N., and Loeschcke V., The Evolutionary and Ecological Role of Heat Shock Proteins, *Ecol. Lett.*, 2003, vol. 6, pp. 1025–1037.
19. Sørensen, J.G., Norry, F.M., Scannapieco, A.C., and Loeschcke, V., Altitudinal Variation for Stress Resistance Traits and Thermal Adaptation in Adult *Drosophila buzzatii* from the New World, *J. Evol. Biol.*, 2005, vol. 18, pp. 829–837.
20. Towbin, H., Staeheln, T., and Gordon, J., Electrophoretic Transfer of Proteins from Polyacrylamide Gels to Nitrocellulose Sheets: Procedure and Some Applications, *Proc. Natl. Acad. Sci. USA*, 1979, vol. 76, pp. 4350–4354.
21. Vepsäläinen, K., Pisarski, B., Kantorek, R., and Laine, K.J., *Formicidae (Hymenoptera)* of Inari Lapland, *Kevo Notes*, 1984, vol. 7, pp. 115–116.
22. Zatsepina, O.G., Velikodvorskaia, V.V., Molodtsov, V.B., Garbuz, D., Lerman, D.N., Bettencourt, B.R., Feder, M.E., and Evgenev M.B., A *Drosophila melanogaster* Strain from Sub-equatorial Africa Has Exceptional Thermotolerance but Decreased Hsp70 Expression, *J. Exp. Biol.*, 2001, vol. 204, pp. 1869–1881.