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Heat shock protein synthesis and thermotolerance in *Cataglyphis*, an ant from the Sahara desert

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ABSTRACT **The ant *Cataglyphis* lives in the Sahara desert and is one of the most thermotolerant land animals known. It forages at body temperatures above 50°C, and the critical thermal maxima are at 53.6 ± 0.8°C for *Cataglyphis bombycina* and 55.1 ± 1.1°C for *Cataglyphis bicolor*. The synthesis and accumulation of heat shock proteins (HSPs) were analyzed in *Cataglyphis* and compared to *Formica*, an ant living in more moderate climates, and to two *Drosophila* species. In *Cataglyphis*, protein synthesis continues at temperatures up to 45°C as compared to 39°C for *Formica* and *Drosophila*. The two *Drosophila* species, *Drosophila melanogaster* and *Drosophila ambigua*, differ with respect to their maximal induction of HSP synthesis and accumulation by 3–4°C. In contrast, the two ant species accumulate HSPs prior to their exposure to heat, and in *Cataglyphis* the temperature of maximal HSP induction by *de novo* protein synthesis is only 2°C higher than in *Formica*. These findings are interpreted as preadaption of the ants prior to exposure to high temperatures.**

In all organisms examined, from Archeobacteria to man, temperature elevation above the normal physiological temperature leads to a *heat shock response*, which consists of a profound alteration of gene expression (reviewed in refs. 1 and 2). As first discovered in *Drosophila* (3), a small number of specific genes, designated as heat shock genes, become actively transcribed under those conditions, while the expression of most of the genes active before heat shock is repressed. The same heat shock genes are also induced by various toxic chemicals and by release from anoxia, indicating that the heat shock response is part of a more general response to various kinds of stress. The heat shock response is a general cellular response since it occurs in all tissues examined and also in isolated cells. Upon returning to the normal temperature, the pattern of gene expression returns to normal. Even if the heat treatment is continued, the heat shock response is transient, but there is a prolonged lag phase before the normal pattern of gene activity is resumed.

The functional significance of the heat shock response is only partially understood, but there is a strong indication that it leads to *thermotolerance* or *thermoprotection*. When *Drosophila melanogaster* larvae are exposed to severe heat shock (40.5°C), the majority of animals die. If, however, a mild heat shock (of about 35°C) is applied immediately before a severe heat shock, about 50% of the animals survive, since they have become thermotolerant or protected (4). The mechanism of thermoprotection is based on the synthesis of specific proteins (heat shock proteins; HSPs) encoded by the heat shock genes. In *D. melanogaster*, the major HSP, HSP70, is encoded by five to seven genes at two closely linked loci, which are largely repressed at normal temperature and strongly induced by heat shock. The *hsp70* genes belong to a gene family, which also includes *hsp68* and seven other genes, so-called heat shock cognates (HSCs), which are strongly expressed at normal temperatures (5, 6). In contrast to HSP70, the HSP83 protein is not only heat inducible, but it is also expressed at relatively high levels during development at normal temperatures. The four small HSPs, HSP22, -23, -26, and -27, are encoded by four genes clustered at the same chromosomal locus. They are heat inducible in all cells but are also expressed in various tissues during development at normal temperatures. In contrast to HSP70 and HSP83, which are highly conserved in evolution, the four small HSPs are more variable among various *Drosophila* species and other insects with respect to their molecular weight (W.J.G., unpublished results).

The HSPs serve a molecular chaperone function (reviewed in ref. 7). The HSP70 proteins have diverse functions in protein folding, translocation across membranes, assembly, and metabolism. Upon heat shock, HSP70 accumulates in the nucleolus, where it presumably binds to hydrophobic regions exposed on unfolded proteins such as pro-ribosomal fragments and serves to solubilize and protect such proteins. ATP-dependent release of the HSP70 chaperones would then allow the substrate protein to refold (8). HSP90, the mammalian homologue of HSP83 in *Drosophila*, seems predominantly involved in negative regulation of proteins like steroid receptors, tyrosine kinases, elongation factor eF-2α, protein kinase C, casein kinases, actin, and tubulin. HSP90 is thought to lock its protein partner in an inactive conformation or to protect phosphorylation sites from activating protein kinases or phosphatases (see ref. 9). The function of the small HSPs, which are related in their amino acid sequence to α-crystallins (10), is not known, but they have been implicated in the organization or protection of the cytoskeleton.

In connection with the chaperone function of the HSPs, it has been proposed that denatured or improperly folded polypeptides are the inducers of the heat shock response (11). This hypothesis is supported by observations on chain-termination mutants in the actin gene of *Drosophila*, which is specifically expressed in the flight muscle. The truncated actin molecules do not assemble properly into muscle fibers, and the heat shock response is specifically induced in the flight muscles at normal temperature (12).

To gain further insight into the heat shock response, we have studied HSP synthesis in an insect, the desert ant *Cataglyphis*, which survives body temperatures of 50°C for at least 10 min (13, 14) and exhibits a critical thermal maximum (CT_{max}) in the range of 53–55°C (ref. 15; R.W., unpublished results). As a control we have used wood ants (*Formica polyctena*), which live in moderate climates, and two species of fruit flies, *D. melanogaster* and *Drosophila ambigua*, in which the heat shock response is induced at different temperatures (W.J.G., unpublished results).

MATERIALS AND METHODS

Animals and Tissue Preparation. The desert ants *Cataglyphis bombycina* and *Cataglyphis bicolor* were collected in

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Abbreviations: HSP, heat shock protein; CT_{max}, critical thermal maximum; HSC, heat shock cognate.

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Maharès (Tunisia; 34.6°N, 8.3°E) and kept in the laboratory for several weeks. The red wood ant, *F. polyctena*, was collected in Uster (Switzerland) by D. Agosti. For the cosmopolitan species *D. melanogaster*, the standard laboratory wild-type stock Oregon-R was used. A stock of the Palearctic species *D. ambigua* was kindly provided by D. Sperlich. The animals were adapted to 25°C for at least 1 week prior to the experiments. For the heat shock treatment, the animals were anesthetized with ether and dipped in 96% ethanol. Then the brains from single ants were dissected in Grace's insect medium. Because of their smaller size, the *Drosophila* head capsules of well-fed 3- to 4-day-old females were only partly opened, so that the brain tissue was exposed to the tissue culture medium.

Heat Shock Treatment and Analysis of Protein Synthesis. To induce the heat shock response, single ant brains and fly heads were incubated at the respective heat shock temperature for 30 min in an Eppendorf tube containing 10 μl of sterile Grace's insect medium without methionine buffered with 20 mM Hepes (using phenol red as a pH indicator), after dilution of five parts of medium with one part of distilled water (see ref. 16). After 30 min, 1 μl of [^{35}S]methionine (NEN; specific activity of 1220 Ci/mmol, concentration of 10.2 mCi/ml; 1 Ci = 37 GBq) was added, and incubation continued at the same temperature for 4 h. Incorporation was stopped by adding 1 ml of balanced saline solution containing 1 mM unlabeled methionine. The supernatant was removed and the tissue was extracted with 30 μl of SDS extraction buffer containing 62.5 mM Tris·HCl (pH 6.8), 2% SDS, 10% (vol/vol) glycerol, 0.001% bromphenol blue, and 5% 2-mercaptoethanol (added just prior to use). The fly heads were homogenized in this buffer. The proteins were separated on SDS/12% polyacrylamide gels by electrophoresis at 40 V overnight. The gel was fixed in 10% (vol/vol) trichloroacetic acid (TCA) for 15 min, dried on a gel dryer at 70°C for 2 h, and exposed to Fuji RX film for 2 weeks at room temperature without any screen. For determining the total ^{35}S incorporation, 1 μl of extract was spotted onto a Whatman GF/C filter presoaked with 200 μl of 5% TCA, precipitated at 4°C for 20 min, washed with 5% TCA, dried with ethanol on a suction flask, and counted in an Emulsifier-safe mixture (Packard) on a liquid scintillation analyzer (Packard 2000 CA). The autoradiograms were digitalized and quantitated on a Vilber Lourmat gel photography system with a Siltron computer using the BIO-PROFIL BIO 1D Version 5.07 software.

HSP Accumulation. The HSP levels were measured by Western blotting using a monoclonal rat anti-HSP70 family antibody (IgG, clone 7.10) obtained from Affinity Bioreagents (17). This antibody recognizes proteins encoded by several HSP70 gene family members including HSP70, -72, -68, and cognate proteins from a wide variety of eukaryotes. Even though it also recognizes cognate proteins (HSC70), which are not heat induced, it can be used to detect heat induction of HSP70 on one-dimensional gels (see below). The living animals were exposed for 4 h to 25°, 37°, and 39°C, respectively, and sacrificed for protein analysis. Ten *Drosophila* and five ant heads were homogenized in sample buffer, and equivalent amounts of extract corresponding to one *Drosophila* head and one-half of an ant head were loaded per gel slot. The proteins were separated on 12% polyacrylamide gels (as above) by electrophoresis of 40 V overnight and electroblotted onto Millipore Immobilon-P membranes (0.45 μm). The membranes were washed in PBS, stained for 15 min in Coomassie brilliant blue, and photographed. Upon destaining for 15 min in destaining buffer and 3 min in 90% methanol, the membranes were washed twice in PBS containing 0.1% Triton X-100 (10 min each) and blocked with 5% Blotto (low-fat dried milk powder) solution in PBT for 20 min. Monoclonal antibody 7.10 was added at a 1:4000 dilution, and the membrane was incubated for 2 h at room temperature. After washing in Blotto, PBT, and 1% blocking solution (Boehringer Mannheim) in PBT, a secondary antibody, horseradish peroxidase-conjugated rabbit anti-rat, was added at a 1:4000 dilution, and the membrane was incubated overnight at 4°C. For detection, the membrane was washed four times in PBT (15 min each), dried, placed on a sheet of plastic, and overlaid with ECL solution (Amersham) according to the instructions by the manufacturer. The membrane was covered with a second plastic sheet and exposed on Kodak x-ray film X-Omat AR for 1 and 5 min, and the film was developed.

Determination of CT_{max}. For measurements of CT_{max}, individual ants were placed into a 100-ml Erlenmeyer flask, which was immersed in a water bath above a heating element. Within this experimental setup, the air temperature of the flask could be raised progressively by 1°C/min. Flask temperature was monitored inside a second flask at ant height (i.e., 4 mm above ground) with a copper-constantan thermocouple linked to a digital thermometer (Bailey Instruments, model BAT-12). The ant's CT_{max} response (curling up of metasoma and thorax, muscle spasms, and jerky leg movements) could be determined unambiguously with an accuracy of 0.1–0.2°C. Essentially the same procedure was used for *Drosophila*. Close to the CT_{max}, fruit flies become incapable of righting themselves after falling on their backs, and subsequently they can no longer move their legs.

RESULTS

HSP Synthesis. Prior to the experimental analysis of protein synthesis, all animals were adapted to 25°C (except for *D. ambigua*, which was maintained at 18°C). To induce the heat shock response, the brain tissue dissected from a single animal was incubated in Grace's insect medium without methionine for 30 min before adding [^{35}S]methionine followed by continued incubation at the same temperature for 4 h.

In the *D. melanogaster* controls (Fig. 1 *A* and *E*), HSP synthesis is first induced weakly at 27°C and at 29°C and more strongly at 31°C; the induction of the four small HSPs, HSP22, -23, -26, and -27, is maximal at 33°C. HSP70 and HSP83 are also most strongly induced at 33°C and continue to be synthesized at 35°C and 37°C, when the synthesis of the small HSPs is strongly reduced. At 35°C and 37°C, it is almost exclusively the synthesis of HSPs that can be detected, whereas the synthesis of most other proteins that are synthesized at 25°C has ceased. At temperatures above 39°C, no protein synthesis is detectable. A labeled protein band detected at temperatures as high as 50°C proved to be a labeling artifact when the culture medium contained [^{35}S]cysteine in addition to [^{35}S]methionine.

In *D. ambigua* the heat shock response is induced at a lower temperature than in *D. melanogaster* and another 30 *Drosophila* species surveyed (W.J.G., unpublished results). The induction of HSP70 is already detectable at 25°C (Fig. 1 *C* and *E*), reaches a maximum at 32°C, and rapidly declines at 35°C. At 39°C protein synthesis is still detectable as in *D. melanogaster*, but the peak of HSP70 induction is shifted by about 4°C.

In *F. polyctena* (Fig. 1 *D* and *F*), there is a much more gradual heat shock response with two bands that comigrate with *Drosophila* HSP83 and HSP70, whereas the homologues of the small HSPs cannot be easily identified. The HSP70 and HSP83 homologues are expressed most abundantly at 33, 35, and 37°C. In contrast to *Drosophila*, the repression of the non-HSPs is rather incomplete at 37°C. A small amount of protein synthesis is detectable up to 39°C.

Similar to *Formica*, *Cataglyphis* shows a very gradual heat shock response, and it seems that HSP70 and HSP83 are already synthesized at 25°C. The intensity of these two bands increases to a maximum around 37°C without much repression of the non-HSPs. In contrast to *Formica*, protein synthesis continues up to 43°C, indicating that *C. bombycina* is considerably more thermotolerant than *F. polyctena*. Similar results

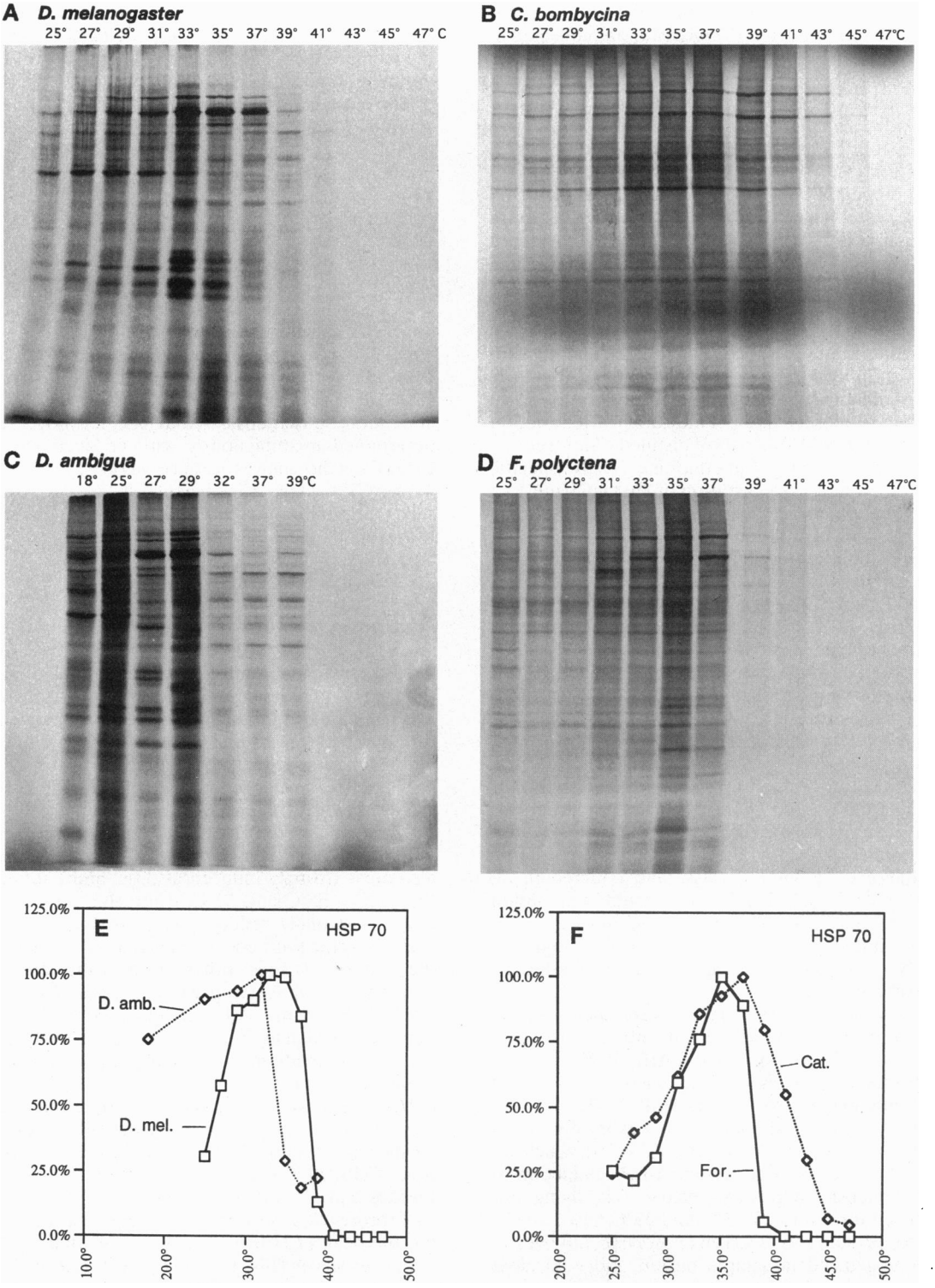

FIG. 1. HSP synthesis at various temperatures in the desert ant *Cataglyphis* as compared to *Formica* and *Drosophila* living in more moderate climates. ^{35}S incorporation and SDS/PAGE analysis of the labeled proteins. (*A*) *D. melanogaster*. (*B*) *C. bombycina*. (*C*) *D. ambigua*. (*D*) *F. polyctena*. Quantitative analysis of HSP70 synthesis, comparing *D. melanogaster* and *D. ambigua* (*E*) and *C. bombycina* and *F. polyctena* (*F*), respectively (see *Materials and Methods*), is shown.

were obtained for *C. bicolor*. Thus, *Cataglyphis* seems to synthesize HSP70 and HSP83 prior to exposure to high temperature and is capable of protein synthesis at significantly higher temperatures than *Formica*.

HSP Levels. The levels of HSP70 were determined by Western blotting with monoclonal antibody 7.10 (see *Materials and Methods*). Even though this antibody detects various members of the HSP70 family, the heat induction of HSP70 can clearly be demonstrated in *Drosophila* (Fig. 2), since the background of noninducible HSC70 is low. In *D. melanogaster* (Fig. 2), the highest levels of HSP70 are reached after a 4-h exposure to 35°C and 37°C, whereas in *D. ambigua* the highest levels were measured at 32°C, confirming the results obtained by ^{35}S incorporation.

Comparison of *D. melanogaster*, *C. bombycina*, and *F. polyctena* shows significant differences (Fig. 3). In *D. melano-*

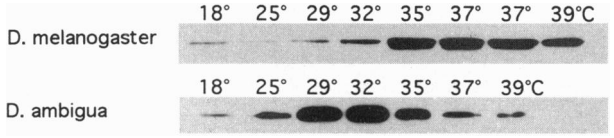

FIG. 2. Western blot of HSP accumulation at various temperatures detected by monoclonal antibody 7.10 (17). Comparison between *D. melanogaster* and *D. ambigua* heads (see *Materials and Methods*) is shown. *D. ambigua* accumulates HSP70 at lower temperatures than *D. melanogaster*. The second 37°C slot for *D. melanogaster* shows the HSP accumulation in the ovary for comparison.

gaster the antibody 7.10 detects both HSP70 and HSP68, which are barely resolved on this gel even though the acrylamide concentration was lowered to 8.25% for this particular experiment. In the two species of ants, two bands at about 70 kDa and 72 kDa are detected. In contrast to *Drosophila* in which the levels of both HSP70 and HSP68 are highly elevated upon exposure to 37°C and 39°C for 4 h, the levels of both the 70-kDa and 72-kDa proteins are almost equal with or without heat treatment in the two ant species, indicating that HSP70 is already expressed at 25°C. Thus, the Western blotting experiments corroborate the conclusion that in *Cataglyphis* and also in *Formica* HSP70 is already induced prior to heat treatment. The two proteins of 70 and 72 kDa can even be detected at similar levels, when *C. bombycina* and *F. polyctena* are taken directly from their nests at 30.1°C and 19.1°C, respectively.

CT_{max}. CT_{max} is defined as the temperature at which the animal is no longer capable of proper locomotion—i.e., of escaping from a "thermal trap". The CT_{max} values for five species analyzed are listed in Table 1. The two *Cataglyphis* species have extremely high CT_{max} values, which correspond to the environmental temperatures encountered in the desert. For *Formica*, the values are significantly lower ($P < 0.001$). The two *Drosophila* species are significantly less thermotolerant than both ant species, and also the difference between *D. melanogaster* and *D. ambigua* is statistically significant: *D. ambigua* is 2.4°C more thermosensitive than *D. melanogaster*. These measurements correlate well with our findings on HSP synthesis and accumulation.

DISCUSSION

Cataglyphis is one of the most thermotolerant land animals known. It can hunt in the desert at body temperatures well above 50°C (15). Similar thermotolerant behavior has been described in *Ocymyrmex* (Myrmicinae) and *Melophorus* (Formicinae) species, which are the ecological equivalents of the *Cataglyphis* species in the Namib and central Australian deserts, respectively (18, 19). Except for these "thermophilic" species, all other desert ants stop foraging when surface temperatures rise above 45°C (reviewed in ref. 20). Hence, desert ants of the genus *Cataglyphis* fill a particular ecological niche. They are neither predators nor harvesters, but scavengers. They forage for the corpses of insects and other arthropods that have succumbed to the heat stress of their desert environment. In the summer months, *Cataglyphis* is the only animal of less than about 1 kg body mass known to be active

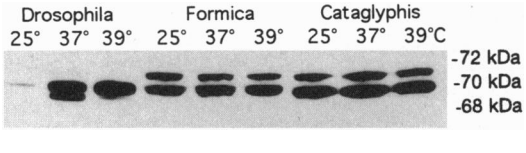

FIG. 3. Comparison of HSP accumulation in *D. melanogaster*, *F. polyctena*, and *C. bombycina*. Note the accumulation of HSP70 and HSP72 in the two ant species at 25°C prior to heat induction.

Table 1. CT_{max}

Species	*n*	CT_{max}, °C
C. bombycina	36	53.6 ± 0.8
C. bicolor	36	55.1 ± 1.1
F. polyctena	36	46.8 ± 1.6
D. melanogaster	13	43.5 ± 0.5
D. ambigua	13	41.1 ± 0.5

n, Number of measurements.

on the Saharan sand surfaces during daytime, even under the hot midday sun. Long legs keep the body at a height of about 4 mm above ground, where temperatures may be >10°C lower than on the sand surface (21), and allow for high running speeds (up to 1 m/sec) (22) and thus short foraging and homing times. In addition, the animals can load off excess body heat by engaging in a particular kind of thermal respite behavior (15).

In spite of all these behavioral adaptations, one wonders whether the animals have adapted to heat-stress conditions of their foraging life both physiologically (23) and biochemically. Thermophilic bacteria, which live at even higher temperatures, are protected against the heat by having much more stable enzymes and other proteins, which become denatured at temperatures that are much higher than those of nonthermophilic bacteria. Therefore, one might expect that enzymes and other proteins are more stable in *Cataglyphis* than in insects living in more mesic habitats.

In *Cataglyphis* we find that protein synthesis continues at temperatures up to 45°C, whereas in the Palearctic sister group of *Cataglyphis*, namely *Formica*, it stops at temperatures above 39°C. If the heat shock response is triggered by denatured proteins (see Introduction), the temperature at which the response is induced should be higher in *Cataglyphis* than in *Formica*. Surprisingly, however, our data show the reverse. In *Cataglyphis*, HSPs, in particular HSP70 and HSP72, are synthesized not only at particularly high temperatures but also at low temperatures. In both *Cataglyphis* and *Formica*, significant levels of HSP70 and HSP72 are accumulated at low temperature, which differs significantly from both *Drosophila* species analyzed. In *C. bombycina*, the whole forager force leaves the nest in a dramatic short-term outburst lasting only for a few minutes (15). Then, within seconds, the ants encounter a shift in body temperature from <30°C inside their nest cavities (24) to often >50°C in their outdoor foraging areas (21). The duration of the outburst is presumably too short for the synthesis of the HSPs, so it may be of selective advantage to presynthesize HSPs prior to the exposure to extremely high ambient temperatures. A similar mechanism of thermoprotection seems to have evolved, albeit less efficiently, in *Formica*, whereas *Drosophila* (Table 1) is much more thermosensitive.

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