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Original article

Induction of heat shock proteins in the larval fat body of *Apis mellifera* L. bees

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Abstract – Five proteins were expressed in larval honey bee fat bod incubated in vitro in response to heat shock, as shown by SDS-PAGE and fluorography. The large heat shock proteins (82, 70 kDa) were inducible throughout the 5th instar whereas the small ones (29, 26, 16 kDa) were inducible only in certain phases of this instar. The synthesis of these HSPs was accompanied by generalized inhibition of overall protein synthesis and secretion in the culture medium. Fluorograms showed that the 76 and 74 kDa proteins were strongly inhibited by heat treatment. Western blots using a mouse monoclonal antibody against HSP72 and HSC73 permitted the inference that the 70 kDa larval protein accumulated in the honey bee fat body in response to heat shock corresponds to the HSP72 isoform. The Western blots also showed a 70 kDa faint band in fat bodies incubated at the control temperature (34 °C). This protein, also detected in incubation media independently of the temperature used, was interpreted as being the constitutively synthesized and secreted HSC73 isoform.

heat shock proteins / HSP70 / *Apis mellifera* / fat body

1. INTRODUCTION

Special proteins, the heat shock proteins (HSPs), are rapidly synthesized when organisms or cultured cells are exposed to elevated temperatures [18, 22, 25]. This so-called heat shock response is a ubiquitous mechanism which helps organisms survive during conditions of stress by repairing or removing damaged subcellular structures and maintaining cell integrity. Most HSPs are members of protein families

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characterized by subunit molecular weights of 20–30, 60–70 and 80–100 kDa [32] and have been studied in a diverse array of organisms including different insect orders, such as Diptera, Lepidoptera, Orthoptera and Dictyoptera [1, 3, 4, 6, 9, 10, 14, 17, 20, 29, 31, 36, 38, 41].

Increased levels of 6 translatable RNAs and induction of HSPs of 82, 70, 36, 33, 27 and 23 kDa were observed in adult *Apis mellifera* worker bees (Hymenoptera) [33] following heat stress at 42 °C for 4 hours. HSP induction with consequent acquisition of thermotolerance has been proposed to be important for worker bees during flight, when their thoracic temperature increases, at times even reaching 47 °C. This response seems necessary also for protecting bees sitting within the bee cluster formed inside the colony during winter in cold climates. In these bees, the thoracic temperature exceeds 40 °C due to microvibration of flight muscles to maintain cluster temperatures of 33–34 °C [35].

Inside the colony, the temperature is normally maintained at around 35 °C through social homeostatic adjustments [35]. The mechanisms of temperature control require cooperation among the adult workers of the colony. Increased water collection and evaporation by fanning wings and formation of wide spacing among workers within the colony are mechanisms that permit maintenance of a stable temperature, preventing egg and larva desiccation and thus assuring the development of the immature forms. Even when the air temperature was as high as 70 °C in hives placed on a lava field under the full sun, the bees were able to keep a normal temperature inside the colony [35]. Thus, the immature forms, i.e. larvae and pupae, continue to be well protected within the colony in the presence of changes in environmental conditions. Such a precise temperature control exerted at the colony level, however, does not prevent the immature stages from evolving their own defense mechanisms against temperature changes, since larvae on the outer edges of colonies might face different temperature variance than more central larvae.

The present study shows that honey bee larvae express HSPs in response to thermal treatment. The program of gene expression in the larval fat body was rapidly altered in response to elevation in temperature. This is demonstrated here by using fat body cells incubated in vitro as a system to study HSPs since temperatures can be readily shifted and controlled. Fat body cells were chosen because they have a generalized function in insect homeostasis. Using SDS-PAGE and fluorography, we identified five heat-induced proteins in larval fat bodies incubated at 42 or 47 °C for 2 hours. Proteins immunologically related to proteins of the HSP70 family, the HSPs most highly conserved, were detected in fat bodies submitted to thermal treatment, as shown by Western blots.

2. MATERIALS AND METHODS

2.1. *Apis mellifera* larvae

Fifth instar feeding larvae (F1 and F2) or larvae that had already completed the feeding period and were starting to spin their cocoon (S1) were collected from colonies of Africanized stocks kept in the experimental apiary of the Department of Genetics, Faculty of Medicine of Ribeirão Preto, State of São Paulo, Brazil. Larval weight was used as an indicator of developmental stages [21].

2.2. Fat body in vitro incubations

F1, F2 and S1 larvae were rapidly immersed in Ringer for insects (0.17 M NaCl, 0.01 M KCl, 0.003 M $CaCl_2$, and 10% sucrose, w/v, containing phenylthiourea crystals). A longitudinal incision was then made in the dorsum of the larvae under a stereomicroscope in an aseptic chamber, and the intestine and Malpighian tubules were removed. The head and the last two

abdominal tergites were discarded, and the fat body mass adhering to the epidermis was incubated in culture medium.

The fat bodies were individually incubated in 1 ml complete culture medium developed for bee larvae [27] for 15 to 40 min at 34 °C, and then transferred to 1 ml of this medium containing 12.5 μCi [^{3}H]L-leucine (147 Ci/mmol, Amersham) where they were incubated for 2 hours at 34 °C (control group) or at 42 °C, under constant shaking (100 rpm/min). Higher temperatures (47 °C and 55 °C) were also tested. In each experiment and for the different developmental phases, we set up in vitro incubations of 6 fat bodies for the control and the same was done for the experimental group. Experiments were repeated at least three times. After incubation, each fat body and its medium were collected separately. The fat bodies were individually macerated in 100 μl 12% sorbitol (w/v) and centrifuged at 5 000 *g* for 5 min at 7–10 °C. Supernatants (fat body extracts) and the medium in which each fat body was incubated were stored at –20 °C before being analyzed for de novo protein synthesis and secretion.

2.3. Protein precipitation

The proteins from aliquots (5 μl) of fat body extract were precipitated with 500 μl 15% TCA in water (w/v), using 10 μl 1% bovine serum albumin (BSA) (w/v) in 0.9% NaCl as carrier. The proteins present in 100 μl aliquots of the incubation medium were also precipitated with 500 μl 15% TCA but in this case, 50 μl of the BSA solution were used as carrier. After centrifugation at 2 000 *g* for 2 min at 7–10 °C, pellets were washed three times with 500 μl 10% TCA.

2.4. Quantification of proteins synthesized and secreted in vitro

The pellets obtained from fat body extracts and their respective incubation media were dissolved in 100 μl tissue solubilizer (Serva), neutralized with 5 μl glacial acetic acid, and mixed with 1 ml scintillation fluid (0.5% 2,5-diphenyl-oxazole in toluol, w/v). Radioactive labels were quantified by liquid scintillation spectrometry (Multi-Purpose Scintillation Counter/ LS 6500 Beckman-USA).

The cpm values obtained for the different larval phases studied were compared using Kruskal-Wallis (ANOVA) or Dunn's method for all pairwise multiple comparisons. Mann-Whitney or *t*-tests were used to compare cpm values obtained from incubations at 34 or 42 °C.

2.5. Sample preparation for SDS-PAGE

Due to low protein concentrations, samples of incubation media were concentrated by acid precipitation as described above. Pellets were neutralized with 1N NaOH and diluted in sample buffer for SDS-PAGE [15] before being submitted to electrophoresis. Fat body extracts were diluted directly in a sample buffer without previous acid precipitation.

2.6. SDS-PAGE

The proteins of fat body extracts and culture media were separated by SDS-PAGE [15] in gradient gels (7–15%) measuring $100 \times 120 \times 0.7$ mm. SDS was omitted from buffers for separating and stacking gels. Electrophoresis was carried out at a constant current of 15 mA at 7–10 °C and the gels were then prepared for Western blotting and fluorography.

2.7. Fluorography

After electrophoresis, the polyacrylamide gels were fixed in glacial acetic acid for 10 min, incubated with 20% 2,5-diphe-nyl-oxazole in glacial acetic acid (w/v) for 90 min, and washed with water [34]. After

drying, the gels were exposed to X-OMAT AR film (Kodak) at –80 °C for 8 to 45 days.

2.8. Western-blotting

For Western-blotting procedures [37], proteins separated by SDS-PAGE were transferred to Immobilon-P membranes (Millipore) with a pore diameter of 0.45 μm. After two washes of 5 min each in TTBS (0.01M Tris/HCl, 0.9% NaCl (w/v), and 0.1% Triton X-100 (v/v), pH 7.6), the membranes were incubated for 30 min at 15 °C with normal rabbit serum diluted 1:50 (v/v) in 5% BSA in TTBS (w/v). The membranes were then incubated at 15 °C for 20 hours with mouse monoclonal anti-HSP70 antibody (Sigma) diluted 1:5000 (v/v) in 5% BSA in TTBS. This antibody recognizes two members of the HSP70 family: HSC73 (the constitutive form) and HSP72 (the induced form). The membranes were then washed five times for 1 min each in 5% skim milk in TTBS (w/v) and incubated for 90 min at room temperature with peroxidase-conjugated anti-mouse IgG (Dako) diluted 1:4000 (v/v) in 5% BSA in TTBS. Finally, the membranes were washed twice for 5 min each in TTBS, twice for 5 min in TBS (0.01 M Tris/HCl, 0.9% NaCl, w/v, pH 7.6), and stained with diaminobenzidine (DAB) (5 mg in 40 ml TBS and 400 μl 3% H_2O_2, v/v). The staining reaction was stopped with water. Some membranes stained with DAB were subsequently treated with 20% PPO in acetic acid (w/v), dried and exposed to X-OMAT AR film at –80 °C for 30–60 days.

3. RESULTS

3.1. Overall protein synthesis was impaired in fat bodies incubated at 42 °C

The quantification of labeled proteins by liquid scintillation spectrometry showed significantly lower rates of protein synthesis in fat bodies incubated at 42 °C. Fat bodies from all larval phases studied synthesized less protein at this temperature when compared to controls (Fig. 1). Protein secretion into the incubation media was also diminished at 42 °C, a reflection of the diminished synthesis at this temperature (Fig. 2).

3.2. Protein synthesis was higher in F2 than in F1 or S1 fat bodies

Figures 1 and 2 also show differences in the rate of protein synthesis and secretion by fat body cells from the different larval phases studied: F2 exhibited the highest levels, independent of the incubation temperature. This result shows that the rate of synthesis is not proportional to fat body or larval size since S1 larvae are larger, weighing on average 124 mg, but synthesize less protein than F2 larvae, which weigh on average 72 mg.

3.3. Five proteins were induced in fat bodies in response to heat shock

Heat shock at 42 or 47 °C induced the synthesis of proteins of 82 and 70 kDa in the fat bodies of all larval phases studied (F1, F2 and S1) (Figs. 3a, 3b). In contrast, induction of small HSPs (29, 26 and 16 kDa) was restricted, occurring only in some of the developmental phases studied. The 29 kDa protein was induced only in the fat bodies of F1 larvae (Fig. 3a) and the 26 kDa protein was synthesized in a clearly augmented manner in phases F2 and S1 (Fig. 3b). The 16 kDa protein appears to be a developmentally regulated protein, which is synthesized by fat bodies of F1 larvae (Fig. 3a) but is no longer produced by fat bodies of the subsequent phases, F2 and S1 (Fig. 3b) incubated at 34 °C. Synthesis of this protein, however, was induced in F2 after exposure of the fat body to 42 °C (Fig. 3b). Table I shows the proteins induced by heat shock during the different larval

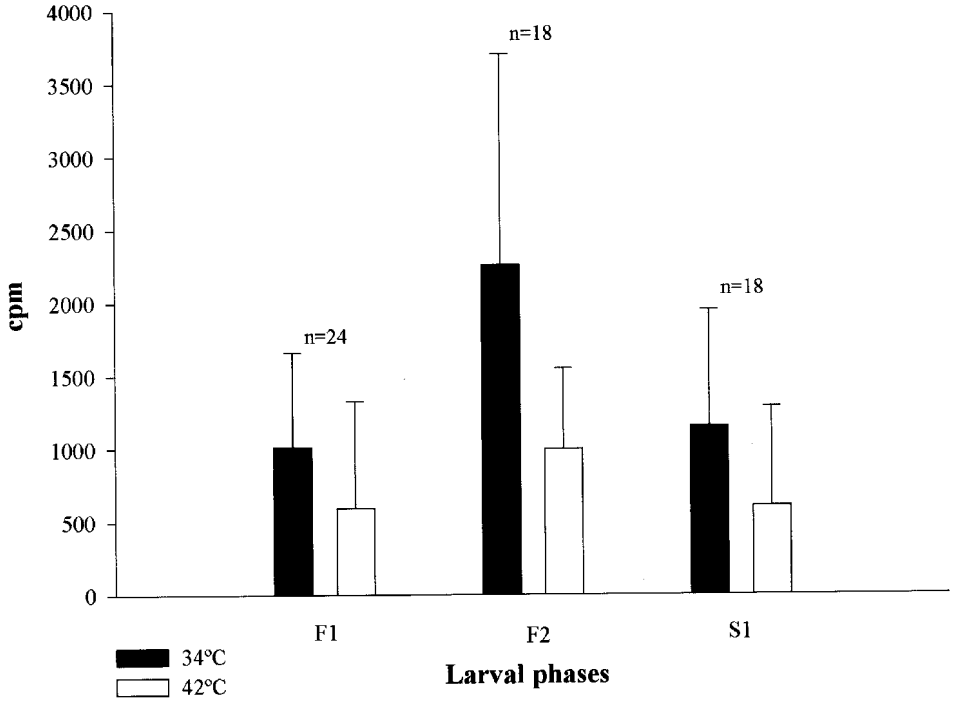

Figure 1. Mean quantity of proteins (cpm) synthesized by the fat bodies of F1, F2 and S1 larvae incubated in vitro at 34 °C or 42 °C. The protein synthesis at 42 °C was significantly lower than at 34 °C for F1 (Mann Whitney, $p = 0.001$), F2 (*t*-test, $p = 0.005$) and for S1 (Mann Whitney, $p = 0.003$) larvae. The rates of protein synthesis were also different between the larval phases studied (Kruskal-Wallis, ANOVA; $p = 0.006$ for comparisons between F1, F2 and S1 incubated at 42 °C; $p = 0.002$ for comparisons between F1, F2 and S1 incubated at 34 °C). Pairwise multiple comparison (Dunn's method) was used to detect the larval phases that differed from each other. It was seen that F1 and F2 synthesized significantly different quantities of proteins at 34 °C or 42 °C ($p < 0.05$); F2 and S1 also differed in synthetic activity in 34 °C-incubations ($p < 0.05$). F1 and S1 were not significantly different ($p > 0.05$).

phases studied. The increased synthesis of the 82, 70, 29, 26 and 16 kDa proteins in response to treatment at 42 °C is evidence that these are HSPs. The elevated temperature also caused depression of the 76 and 74 kDa proteins which were barely detectable in fat bodies incubated at 42 °C (Figs. 3a, 3b).

3.4. Identification of developmentally regulated proteins

The study of the three different phases of the fifth instar permitted the identification of developmentally regulated proteins (Figs. 3a, 3b). Synthesis of a 180 kDa protein, which is quite pronounced in F1 larvae, declined in F2 and was no longer detected during the subsequent phase, S1. In contrast, synthesis of a 150 kDa protein was more evident in the F2 and S1 phases. The 114 kDa protein seemed to be increased in S1, but the secretion pattern (Fig. 4a) did not support this evidence. The 26 and 16 kDa HSPs are also developmentally regulated. By comparing incubations of F1, F2 and S1 fat bodies at 34 °C it could be

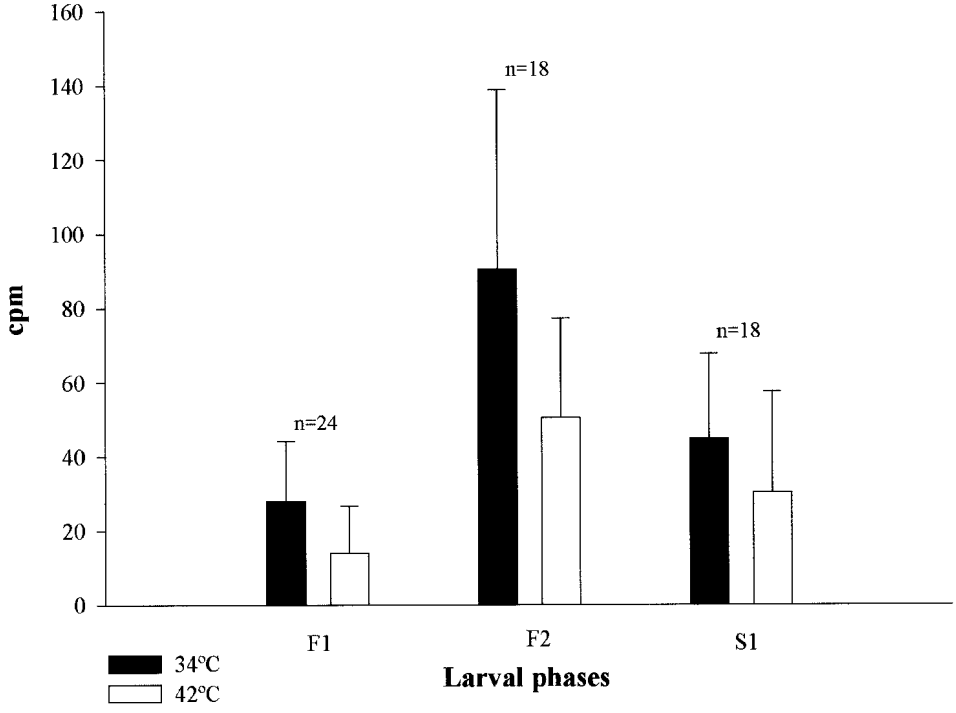

Figure 2. Mean quantity of proteins secreted by fat bodies of F1, F2 and S1 larvae incubated in vitro at 34 or 42 °C. At 42 °C, the protein secretion by F1 and F2 fat bodies was significantly lower than at 34 °C (F1, $p < 0.001$; F2, $p = 0.004$, Mann Whitney test and t-test, respectively). The elevated temperature did not affect the rate of protein secretion by S1 fat bodies ($p = 0.093$, *t*-test). Comparisons between larval phases showed that they secreted different quantities of proteins into the incubation media, independently of the temperature used (Kruskal-Wallis, ANOVA; $p < 0.001$ for comparisons between F1, F2 and S1 incubated at 34 °C or 42 °C). Pairwise multiple comparison (Dunn's method) was used to detect the larval phases that differed from one another. It was seen that F1 and F2 secreted significantly different quantities of proteins at 34 °C or 42 °C ($p < 0.05$), and F2 and S1 also differed in protein secretion in 34 °C-incubations ($p < 0.05$). F1 and S1 did not differ significantly ($p > 0.05$).

observed that the synthesis of the 26 kDa protein decreased from F1 to S1, and that the 16 kDa protein, shown by F1 larvae, was almost undetectable in F2 and S1 larval fat bodies (Figs. 3a, 3b).

Table I. Proteins induced by heat shock in fat bodies from 5th larval instar (F1, F2 and S1 phases) incubated at 42 °C. (–) indicates non-induced protein.

Phases of the 5th larval instar	Protein molecular weight (kDa)				
F1	82	70	29	–	–
F2	82	70	–	26	16
S1	82	70	–	26	–

3.5. In general, heat shock proteins were not secreted into the incubation medium

Acid precipitation of aliquots of the incubation media followed by SDS-PAGE and fluorography permitted us to concentrate and identify the proteins secreted by the fat bodies of F1, F2 and S1 larvae. Figure 4a shows that proteins of 150, 114, 76, 74, 61.4

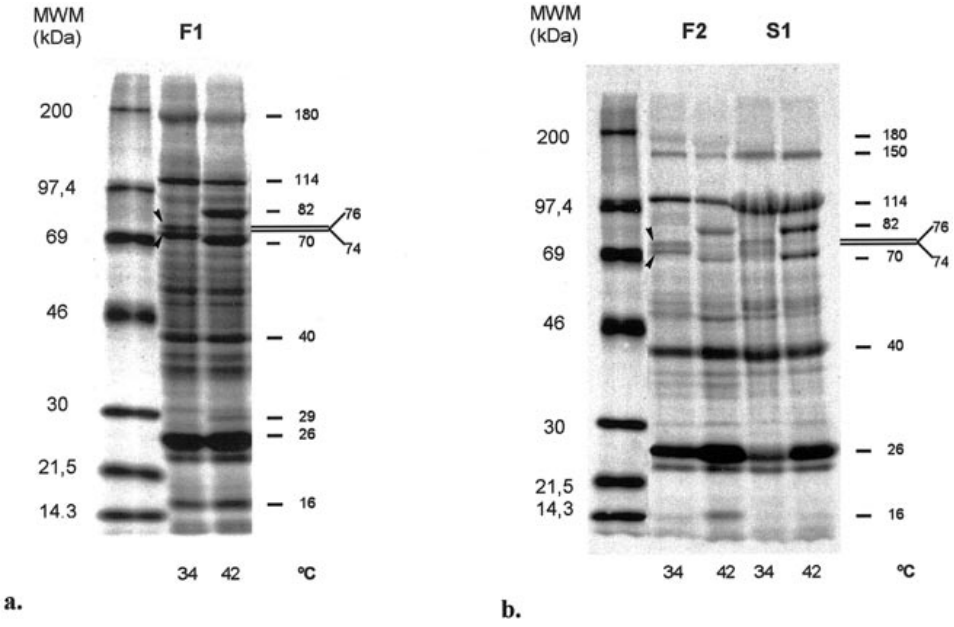

Figure 3. Proteins synthesized by **(a)** F1 larval fat bodies or by **(b)** F2 and S1 larval fat bodies incubated in vitro at 34 °C (controls) or 42 °C. SDS-PAGE (7–15%) followed by fluorography of fat body samples with 8 000 cpm. MWM – molecular weight markers. Numbers at the right of figures indicate relative molecular weight of larval fat body proteins. Arrowheads point to 74 and 76 kDa proteins.

and 16 kDa were secreted into the incubation medium. The 76 and 74 kDa proteins could be seen relatively well separated in Figure 4b, after using more diluted samples of culture media where fat bodies were incubated. Figure 4b clearly shows that the 76 and 74 kDa proteins were secreted in the incubation medium at a lower rate at 42 °C than at 34 °C. The decreased secretion of these proteins at 42 °C (Fig. 4b) reflects their decreased synthesis in the fat body cells at this temperature (Figs. 3a, 3b).

The pattern of secretion of the 150 kDa protein (Fig. 4a) confirmed that the synthesis of this developmentally regulated protein is more intense during the older larval phases, as shown in Figures 3a and 3b. Comparison of Figures 3a, 3b and 4a shows that, except for the 16 kDa protein, the other HSPs (82, 70, 29 and 26 kDa) were secreted only in very small quantities or not at all, indicating that they remained stored in fat body cells.

3.6. Bee larval proteins recognized by antibody against proteins of the HSP70 family (anti-HSP72/HSC73)

A mouse monoclonal antibody against bovine proteins of the HSP70 family recognized the honey bee fat body protein band migrating at the 70 kDa region in Western blots. This protein band appears enlarged and much more intense in 42 °C-treated fat body samples than in controls, indicating increased synthesis in response to the thermal shock. Higher temperature (47 °C) had an even more marked effect. At 55 °C the response was partially inhibited, suggesting a failure in cell functioning (Fig. 5a). The fluorogram of the gel used for Western

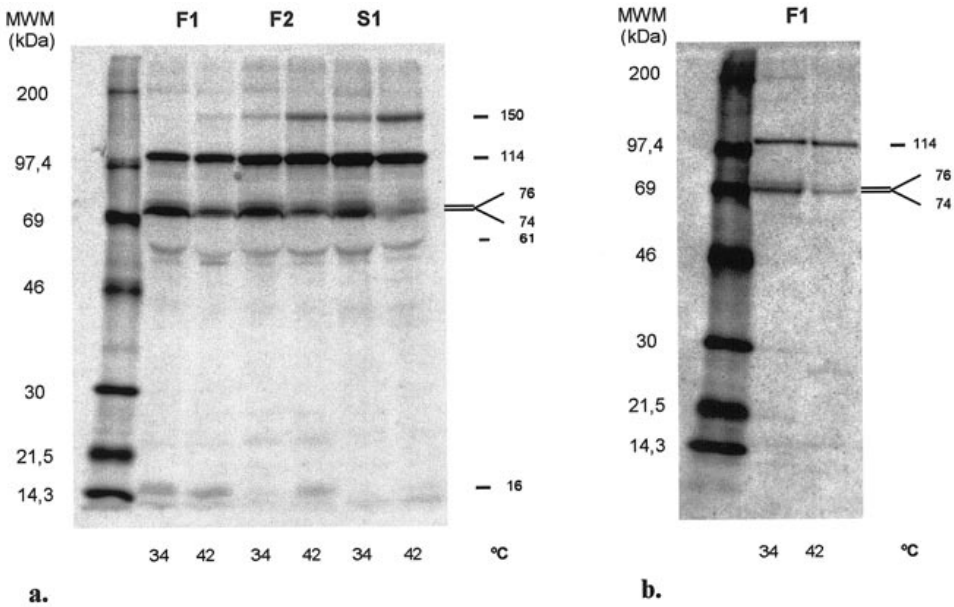

Figure 4. Proteins secreted into the culture medium by fat bodies from F1, F2 and S1 larvae incubated in vitro at 34 °C (controls) or 42 °C. SDS-PAGE (7–15%) followed by fluorography of incubation medium samples containing **(a)** 3 000 cpm or **(b)** 400 cpm. MWM – molecular weight markers. Relative molecular weight of secreted fat body proteins are indicated by numbers at the right of the figures.

blotting (Fig. 5b) permitted deduction of the molecular weight of this protein band by comparison to the radioactive marker. In this figure, the 70 kDa band appears as a white band due to the DAB reaction utilized to identify the protein-antibody complex in the blot. DAB quenched the impression of the X-ray film by the radioactive protein. Although heat shock provoked an increase in the 70 kDa band, denoting augmented protein synthesis in fat body cells (Figs. 5a, 5b), it did not affect the electrophoretic pattern of the culture media where fat bodies were incubated (Fig. 5c), indicating that to an augmented protein synthesis did not correspond an augmented protein secretion. Figure 5 also shows that the antibody used in Western blots recognizes the larval 70 kDa band also in fat body samples incubated at 34 °C (Figs. 5a, 5b), and in their correspondent incubation medium (Fig. 5c); although in both, only a faint activity had been observed. In conjunct these results permitted the inference that the 70kDa band, detected in fat body cells incubated at 34 °C (Figs. 5a, 5b) is the constitutive isoform HSC73. The strong protein band observed in fat body cells incubated at 42 °C (Figs. 5a, 5b) would then correspond to the induced HSP72, comigrating with the constitutive HSC73. Although hardly visualized, the constitutive, but not the induced isoform, is secreted into the incubation medium, albeit in very small quantities, by controls as well as by heated fat bodies (Fig. 5c).

Evidence of the existence of these two larval HSP forms at the 70 kDa region is shown by Western blots of fat bodies shocked at 42 °C (Fig. 6, lane a), in which two protein bands were clearly identified. The corresponding fluorogram (Fig. 6, lane b) permitted comparison of these

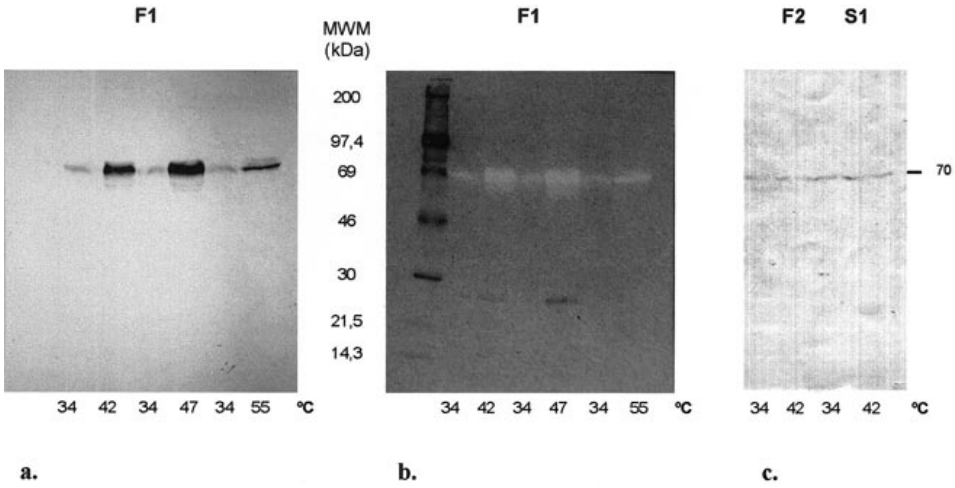

Figure 5. (a) Western blotting using mouse monoclonal antibody against bovine HSP70 protein family (anti-HSP72/HSC73), stained with DAB. Fat body samples from F1 larvae, incubated at 34, 42, 47 or 55 °C. All samples contained 800 cpm. (**b)** A fluorogram of the blot in Figure 5a shows the correspondence in relation to the molecular weight between HSP from F1 larvae and the molecular weight marker (MWM) ~ 70 kDa. **(c)** Western blotting using samples (3000 cpm) of culture medium in which the fat bodies were incubated. The number at the right of Figure 5c indicates relative molecular weight of the larval fat body protein.

proteins to the radioactive ~ 70 kDa molecular weight marker (Fig. 6, lane c). The relative mobility of these proteins at the 70 kDa region of polyacrylamide gels and their response to anti-HSP72/HSC73 antibody suggest strongly that they are the induced (72 kDa) and the constitutive (73 kDa) HSP forms.

4. DISCUSSION

HSP synthesis was induced by heat shock in fat bodies of *A. mellifera* larvae incubated in vitro. HSPs of 82, 70, 29, 26 and 16 kDa were identified by SDS-PAGE and fluorography. At the 70 kDa region, two protein bands were immunologically recognized by a mouse monoclonal antibody against bovine constitutive (HSC73) and inducible (HSP72) proteins, indicating similar structural properties and homology of these proteins between long-diverged animal species. The evolutionary conservation of proteins from the HSP70 family is notorious and argues for an essential role of them in maintaining structural integrity of metazoan cells [2, 32].

Induction of HSP82 and HSP70, the heavier HSPs detected by us in larvae, has been also reported in adult honey bee workers maintained at 42 °C for 4 hours [33]. But the small HSPs induced in larvae, differed in molecular weight from those detected in adult bees. In larvae and in adult bees, different sets of genes might be activated in response to heat shock, resulting in the expression of distinct HSPs, probably reflecting peculiar physiological conditions of distinct developmental stages. Thus, different HSPs could be expressed during development. Examples of variable heat shock expression during the normal course of development and differentiation are well documented [18]. The larval fat body – site of synthesis of the HSPs studied by us –

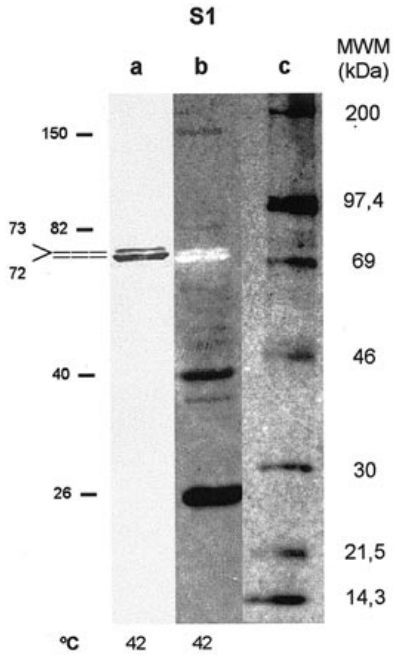

Figure 6. HSP72 and HSC73 in S1 larval fat body cells, incubated at 42 °C. Western blotting using mouse monoclonal antibody against bovine HSP72/HSC73, stained with DAB **(lane a)**, and its corresponding fluorogram **(lane b)**: samples containing 1000 cpm. Radioactive molecular weight markers (MWM) **(lane c)**. Numbers at the left of the figure indicate relative molecular weights of larval fat body proteins detected by Western blotting (lane a) or fluorography (lane b).

shows structural and metabolic properties that are different from adult fat body. The larval fat body can, thus, be expected to respond differently to thermal stress by synthesizing specific sets of HSPs. On the other hand, larvae and adult bees show small HSPs very similar in size and the homology between some of them can not be excluded. Using specific antibodies and comparing sequences of these proteins certainly will contribute to establishing the identities of some of these small proteins as well as their homology in relation to HSPs from other insects.

A common event occurring in cells submitted to heat shock is the interruption of synthesis of some proteins [24]. In the fat bodies of *A. mellifera* larvae, there was a reduction in the synthesis of the 76 and 74 kDa proteins, while the HSPs were simultaneously induced by heat treatment, a fact that contributed to the characterization of the response to stress. The reduction of the 76 and 74 kDa protein synthesis in response to the 42 °C shock, however, cannot be seen as the single cause for the significant decay in the total protein synthesis and secretion in 42 °C-treated fat bodies (Figs. 1 and 2). More probably, the synthetic process as a whole should be affected by the elevated temperature and this was not shown by the fluorograms because the shocked and control samples were prepared in such a way that all of them had the same radioactivity (cpm).

Except for the S1 phase, the decreased rate of protein synthesis at 42 °C resulted in decreased secretion into the culture media (Figs. 1 and 2). Larvae at the spinning phase (S1) suffer intense changes in preparation for metamorphosis. The different response of S1 larvae in terms of rate of protein secretion may be due to the particular physiological conditions of this developmental period.

The HSP70 family often include constitutive as well as heat-inducible protein forms. The antibody used by us recognizes two proteins of the HSP70 family, the induced 72 kDa and the constitutive 73 kDa forms. The protein recognized by this antibody in fat body cells incubated at normal temperature (34 °C) apparently corresponds to the HSC73 constitutive form (Figs. 5a, 5b), which is secreted (to the incubation medium) in small amounts by control as well as by 42 °C-shocked fat bodies (Fig. 5c). Immunoblots of fat bodies incubated at 42 °C showed a broad protein band which seems to correspond mainly to the induced HSP72, comigrating with the constitutive HSC73 (Figs. 5a, 5b). The rise of

HSP72 in fat body cells was not accompanied by a corresponding rise in the culture media (Fig. 5c), evidencing that the induced HSP72 was not secreted. None of the other HSPs synthesized in response to the 42 °C heat shock, except the small 16 kDa heat-induced protein, were secreted into the incubation medium. It is possible that these proteins are most effective within the fat body cells themselves, by conferring protection on them against the harmful effects triggered by the heat shock or acting on cell function repair.

The understanding of the various roles of HSPs in insect cell function and the determination of the temperature limits withstood by some of them are of great practical utility in the control of pests that infest stored products [16]. In practice, treatments with hyperthermia have been used to combat the mite *Varroa jacobsoni* which parasitizes *A. mellifera* bees. When combs containing pupae of drones infested with *V. jacobsoni* were submitted to temperatures of 42–43 °C for 2 hours the infestation was reduced, while the drone pupae were not affected by this treatment [8]. Furthermore, HSPs definitely played an important role in the thermotolerance shown by the drone pupae.

Besides protecting cells against temperature variations, HSPs have other protective functions. The involvement of these proteins in protecting cells from pathological stresses, such as viral infections [40], and other conditions altering cell physiology such as exposure to ethanol, heavy metals, and oxygen free radicals, has been well established [5, 19]. It should also be pointed out that several HSPs have a chaperone function, modulating the folding and unfolding of proteins and facilitating assembly and disassembly of multisubunit complexes [25].

The roles of constitutive HSPs in cell physiology have been reviewed [7, 11, 39]. Some of them are involved in the ecdysone response cascade [23] and, consequently, they act on cell proliferation and differentiation. A heat shock induced 29 kDa protein from ovaries of *A. mellifera* worker larvae responds to ecdysteroids [12]. Transcription of HSP genes in *Drosophila* has also been shown to be inducible by ecdysteroids [13], and steroid receptor-binding sequences were recognized in the promoter regions of HSP genes of this dipteran [28]. There is evidence that HSP90 plays a biologically important role in steroid receptor activity. It has been proposed that this HSP may function in the transport of steroid receptors through the cytoplasm to the nucleus [26]. Other hormones with a function in insect development also affect HSP expression. The stimulatory action of the prothoracicotropic hormone on the synthesis of an HSP70 constitutively expressed in the prothoracic glands has been demonstrated in *Manduca sexta* [30]. Thus, the function of HSPs in the hormonal regulation of the processes of development and differentiation and in cell metabolism clearly indicates the various roles these proteins play, in addition to their protective activity against variations in temperature.

The protective function of HSPs against variations in environmental temperature must be little required, if at all, by honey bee larvae, which develop in extremely stable conditions of temperature and relative humidity. This supports the suggestion that HSPs perform other essential functions of vital importance for bee larvae and certainly also for the larvae of other social insects.

The possible involvement of morphogenetic hormones in the expression of HSPs will be the subject of future research. A first approach to this subject would consist of studying the expression of the constitutive protein HSP73 during pre-imaginal bee development and to relate it to the fluctuations of the hemolymph titers of juvenile hormone and ecdysteroids.

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Résumé – Induction de protéines de choc thermique dans le corps adipeux des larves d'*Apis mellifera* L. L'expression de protéines en réponse à un choc thermique a été étudiée in vitro dans le corps adipeux de larves d'abeilles domestiques, *Apis mellifera* L., placées en étuve à 42, 47 et 55 °C durant 24 h en présence de [^{3}H]L-leucine. Les protéines induites à ces températures ont été étudiées dans le corps adipeux de larves F1 et F2, qui sont aux phases initiales d'alimentation du 5^e stade larvaire, et celui de larves S1, qui ont terminé de s'alimenter et commencé à filer leur cocon (phase filante). Pour chaque expérience et pour les 3 phases larvaires, nous avons procédé à l'incubation in vitro de six corps adipeux pour le groupe témoin et d'autant pour le groupe expérimental. Les expériences ont été répétées au moins trois fois. L'incubation des témoins a été faite à 34 °C, température normalement maintenue au sein de la colonie. Après l'incubation, on a récolté séparément les corps adipeux et leur milieu. Des parties aliquotes d'extraits de corps adipeux et du milieu d'incubation ont été concentrées par précipitation acide. Les protéines radioactives synthétisées de novo par les cellules du corps adipeux et sécrétées dans le milieu d'incubation ont été quantifiées par spectrométrie à scintillation liquide et identifiées par SDS-PAGE et fluorographie. La technique du « Western blotting », utilisant un anticorps monoclonal de souris qui reconnaît deux membres de la famille HSP70, les isoformes HSC73 et HSP72, a été utilisé pour caractériser les protéines de choc thermique chez les larves d'abeilles.

Bien qu'on ait observé une diminution significative de la synthèse et de la sécrétion totales de protéines dans les corps adipeux incubés à 42 °C (Figs. 1 et 2), les résultats ont montré que cette température induit la synthèse de cinq protéines dans les corps adipeux des larves. Deux d'entre elles, de 82 et 70 kDa, ont été induites à toutes les phases larvaires (F1, F2 et S1). Les trois autres protéines plus petites n'ont été induites qu'à certaines phases larvaires : la 29 kDa n'est apparue que chez les larves F1, la protéine 26 kDa chez les larves F2 et S1 et la protéine 16 kDa chez les larves F2 (Figs. 3a et 3b). À l'exception de cette dernière, les protéines induites par la chaleur n'ont pas été identifiées dans le milieu d'incubation (Fig. 4a). Ceci montre qu'elles n'ont pas été sécrétées mais sont restées stockées dans les cellules du corps adipeux, protégeant probablement les cellules des effets nocifs des températures élevées.

Les protéines de choc thermique 26 et 16 kDa sont régulées par le développement. En comparant l'électrophorèse des corps adipeux des stades F1 , F2 et S1 incubés à 34 °C (Figs. 3a, 3b et 4a) on peut voir que la synthèse de la protéine 26 kDa est plus élevée en F1 qu'en F2 et inférieure en S1 ; la protéine 16 kDa, présente dans le corps adipeux de F1, est pratiquement indécelable dans ceux de F2 et de S1. D'autres protéines, régulées par le développement, ont été également notées : la protéine 180 kDa, bien visible au stade F1 seulement et la protéine 150 kDa bien évidente aux stades F2 et S1. La synthèse de ces deux protéines n'est pas affectée par le choc thermique.

Une température élevée, tout en induisant des PCT, réduit simultanément la synthèse et la sécrétion des protéines 76 et 74 kDa (Figs. 3a, 3b et 4a, 4b), ce qui contribue à caractériser la réponse au stress thermique.

Un anticorps monoclonal de souris contre l'isoforme HSP70 constitutive bovine (73 kDa) et l'isoforme HSP70 inductible (72 kDa) (Figs. 5 et 6) a reconnu les protéines correspondantes dans le corps adipeux larvaire. Ceci indique des propriétés structurales similaires et une homologie entre les protéines de choc thermique des larves d'abeilles et celles des mammifères.

protéine choc thermique / HSP 70/ *Apis mellifera* / corps adipeux

Zusammenfassung – Induktion von Hitzeschock – Proteinen im Fettkörper von Larven der Honigbienen (*Apis mellifera* L.). Als Schutz gegen Überhitzung werden von vielen Organismen spezielle Eiweisse gebildet. Diese Hitzeschock – Proteine können zerstörte Zellstrukturen entfernen oder wieder aufbauen und so zum Überleben beitragen. Die Synthese von Proteinen als Reaktion auf einen Hitzeschock von *Apis mellifera* wurde im Fettkörper untersucht, die in Vitro unter Anwesenheit von [^{3}H]L-Leucin für 2 Stunden auf 42, 47 und 55 °C erhitzt wurden. Proteine, die bei diesen Temperaturen erzeugt wurden, wurden in den Fettkörpern von F1 und F2 Larven, am Beginn der Fressphase des 5. Larvenstadiums und von der S1 Larve am Ende Fressphase bzw. Beginn der Spinnphase untersucht. Bei jedem Versuch und bei allen 3 Larvenphasen setzten wir eine Inkubation von 6 Fettkörpern an, sowohl bei der Kontroll- als auch bei der Versuchsgruppe. Die Versuche wurden mindestens 3 Mal wiederholt. Die Inkubation der Kontrolle wurde bei 34 °C durchgeführt, die Temperatur, die normalerweise im Bienenvolk herrscht. Nach der Inkubation wurde jeder Fettkörper und sein Medium getrennt gesammelt. Aliquots der Fettkörperextrakte und des Inkubationsmedium wurden mit Säure ausgefällt. Die in den Fettkörpern neu synthetisierten radioaktiven und in das Medium ausgeschiedenen Proteine wurden mit der Flüssigkeit – Szintillation Zählung quantifiziert und mit SDS-PAGE und Fluorographie identifiziert. Western Blotting unter Anwendung von monoklonalen Antikörpern aus Mäusen, die zwei Gruppen der HSP70 Familien erkennt, die Isoformen HSC73 und HCP72, wurde eingesetzt, um die Hitzeschock – Proteine in Bienenlarven zu charakterisieren.

Obwohl sich bei der Inkubation der Fettkörper bei 42 °C eine signifikante Abnahme bei der Gesamtsynthese von Proteinen und bei ihrer Sekretion ergab (Abb. 1 und 2), konnte bei dieser Temperatur die Synthese von 5 Proteinen im Fettkörper nachgewiesen werden. Zwei von ihnen, das 82 und 70 kDa Protein, wurde in allen Larvalstadien (F1, F2 und S1) induziert. Die 3 kleineren Proteine wurden nur in einigen dieser Stadien induziert: das 29 kDa kam nur bei F1, das 26 kDa bei F2 und S1 vor, während das 16 kDa Protein in der F2 Phase induziert wurde (Abb. 3a, 3b). Mit Ausnahme vom 16 kDa Protein wurde keines der anderen Proteine im Medium gefunden (Abb. 4a). Das bedeutet, dass sie nicht sezerniert sondern in den Fettkörperzellen gespeichert wurden, um diese vor gefährlichen Folgen durch hohe Temperaturen zu schützen.

Während der Larvalentwicklung ist die Bildung von den 26 und 16 kDa Hitzeschock Proteine reguliert. Beim Vergleich des elektrophoretischen Musters von F1, F2 und S1 des bei 34 °C inkubierten Fettkörpers ist zu erkennen, dass die Synthese des 26 kDa Proteins höher in F1 als in F2 und kleiner als in S1 war. Das 16 kDa Protein vom F1 Fettkörper war in F2 und S1 Fettkörpern fast nicht zu erkennen. Andere entwicklungsabhängige Proteine wurden ebenfalls entdeckt, wie z.B. das 180 kDa Protein, das nur in der F1 Larve deutlich zu erkennen ist und das 150 kDa Protein, das in F2 und S1 deutlich sichtbar ist. Die Synthese dieser beiden Proteine ist unabhängig vom Hitzeschock.

Bei der Induktion von HSPs bewirkt die erhöhte Temperatur eine Reduktion der Eiweissynthese bei gleichzeitiger Produktion der 76 und 74 kDa Proteine (Abb. 3a, 3b und 4a, 4b), eine Erscheinung, die einen Beitrag zur Charakterisierung der Reaktionen auf Hitzestress leistet.

Monoklonale Antikörper aus Mäusen gegen das Stammprotein 73 kDa und die induzierbaren (72 kDa) HSP70 Isoformen (Abb. 5 und 6) vom Rind erkannte die entsprechenden Proteine des larvalen Fettkörpers. Das weist auf eine strukturelle Ähnlichkeit und eine Homologie zwischen Hitzeschock – Proteinen aus Bienenlarven und Säugetieren hin.

Hitzeschock–Proteine / HSP70 / *Apis mellifera* / Fettkörper

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