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QUARTERLY

Influence of low temperature on the synthesis of some *Galleria mellonella* proteins*

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In developing *Galleria mellonella* larvae (reared at 30°C) three proteins of 74, 76 and 81/82 kDa were identified. They represent a group of storage proteins (LHP proteins). In *Galleria* larvae, the development of which is arrested by low temperature (18°C), accumulation of the 74, 76 and 81/82 kDa proteins was detected in the hemolymph. The synthesis of 74 kDa and 76 kDa proteins started after 24 h, and that of about 80 kDa after 96 h following the transfer of larvae from 30°C to 18°C. 20-Hydroxyecdysone inhibited synthesis of the 74 and 76 kDa proteins in larvae exposed to low temperature. The arrest of development of *Galleria* larvae is associated with the synthesis and accumulation of storage proteins, and ecdysteroids are involved in these processes.

Insect diapause is an adaptation for survival in unfavourable environment by entry into a state of arrest in reproduction and morphogenesis. Low rates of protein synthesis are characteristic of diapausing insects [1]. On the other hand, in some insects, synthesis of selected proteins has been observed [1]. These diapause-associated proteins are present in insects that exhibit diapause at the embryonic, larval, pupal or adult stages [1–6]. They have been found to arise in the fat body and to accumulate in the hemolymph of diapausing insects. Some of them, however, are not fully specific for diapausing animals as they have also been found in their non-diapausing counterparts [1, 2, 7, 8].

Molecular mechanisms of insect diapause regulation are still unknown. The presence of diapause specific hormone patterns suggests that diapause is a state with characteristic patterns of gene expression [9].

Development of wax moth *Galleria mellonella* is strongly affected by suboptimal rearing temperature [10–12]. When one day old last instar *Galleria* larvae are transferred from optimal temperature (30°C) to 18°C, their development is arrested [12]. According to Mikołajczyk & Cymborowski [12], this arrest of development of *Galleria* larvae might be considered as diapause.

This paper describes investigations on the influence of low temperature on the synthesis of proteins in *Galleria* larvae.

MATERIAL AND METHODS

Insects. Wax moth *Galleria mellonella* (Lepidoptera, Pyralidae) was reared in constant dark-

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Abbreviations: LHP, larval hemolymph proteins.

ness at 30°C on semi-artificial diet prepared as described by Sehnal [13]. The time of 20–24 h after ecdysis of the last (7th) instar larvae was regarded as day 1, 24 h later as day 2 and so on.

One day old last instar larvae were transferred from 30° to 18°C in groups of about 10 individuals and subsequently reared continuously at this temperature. Under these conditions the larvae ceased to develop and never pupated [10].

Fat body and hemolymph proteins preparation. Fat body and hemolymph were obtained from larvae kept at 18°C as well as at 30°C. Fat body was dissected into *Galleria* Ringer solution (130 mM NaCl, 1.3 mM KCl, 1.3 mM $CaCl_2 \times 2H_2O$, 2.0 mM $MgCl_2 \times 6H_2O$, 2.3 mM $NaHCO_3$, pH 6.8). Following several rinses in Ringer's the tissue was homogenized in 50 mM potassium phosphate buffer, pH 8.0, containing 1 mM phenylmethyl sulfonyl fluoride, 1 mM dithiothreitol, 1 mM EDTA and 0.01% 1-phenyl-2-thiourea [14]. After centrifugation of the crude extract for 5 min at 5000 × *g*, the supernatant was stored at –20°C.

Hemolymph was collected with a syringe, immediately diluted 1:10, v/v, with the homogenization buffer and centrifuged at 1000 × *g* for 5 min to remove cellular material. The samples were stored at –20°C.

Protein assay. Proteins of the fat body as well as the hemolymph were estimated by the method of Bradford [15], using bovine serum albumin as a standard.

Preparation of labelled proteins. The proteins were labelled *in vivo* using [^{14}C]leucine (Institute for Research and Application of Radioisotopes, Czech Republic). Larvae were injected intra-abdominally with 5 µCi of [^{14}C]leucine (sp. act. 8.88 MBq/mmol) using a 10 µl syringe. Fat body and hemolymph were collected after 3 or 6 h of incubation with the isotope at 30°C or 18°C, respectively. [^{14}C]Leucine labelled protein samples were prepared as described above. 5 µl of radiolabelled protein extract was spotted on Whatman GF/C filter paper, precipitated with trichloroacetic acid and counted in LKB 1209 scintillation counter. Equal amounts of radioactive material were loaded into each slot of the gel.

Electrophoresis. The proteins were separated on a 7.5% or 10% polyacrylamide gel under denaturing conditions [16]. Prior to electrophoresis, samples were mixed with sample buffer and heated for 3 min at 100°C. Gels were stained for proteins with 0.1% Coomassie Brilliant Blue R-250.

Stained gels containing ^{14}C-labelled proteins were treated with ENHANCE (New England Nuclear Co.) and then dried in vacuum. Dried gels were exposed to Kodak X-ray film [17]. The fluorograms were developed after appropriate exposure at –70°C.

RESULTS AND DISCUSSION

Analysis of hemolymph proteins of developing *Galleria mellonella* larvae (reared at 30°C) was performed using 10% polyacrylamide gel.

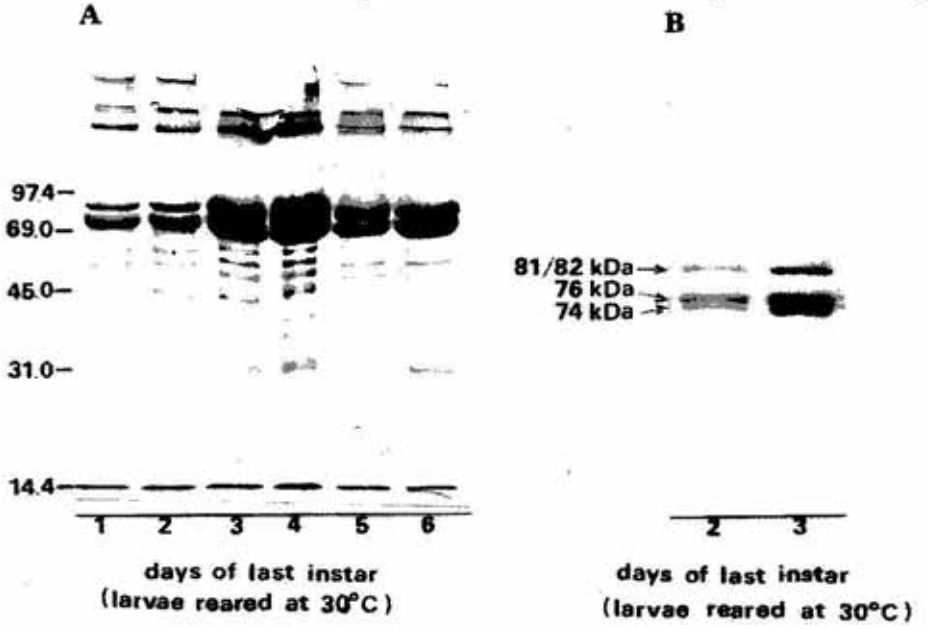

Fig. 1. Changes in the electrophoretic pattern of larval hemolymph proteins during last instar Galleria larvae.
Proteins were analysed in 10% polyacrylamide gel (A) and 7.5% polyacrylamide gel (B). Proteins in 0.5 µl of hemolymph were loaded into each slot of the gel. The size of marker proteins is expressed in kilodaltons.

Figure 1A shows the group of dominant proteins of molecular mass from about 74 kDa to 82 kDa. The content of these proteins increased markedly from day 3 of the last instar, and persisted at a high level until at least day 6. In order to determine the number of distinct proteins in *Galleria* hemolymph, the samples were analyzed on 7.5% polyacrylamide gel. Under these conditions at least three proteins of 74, 76 and 81/82 kDa were identified (Fig. 1B).

Earlier Miller & Silhacek [14] have identified in developing *Galleria* larvae four larval hemolymph proteins (LHP) of 74, 76, 81 and 82 kDa as storage proteins. The proteins are synthesized in the fat body of mid-last instar larvae, released into the hemolymph and then reabsorbed by the fat body into storage granules during the prepupal stage [14, 18, 19].

Our results confirmed the above findings. The data presented in Fig. 2 show that, in the fat body, the synthesis of proteins of molecular mass from about 74 kDa to 82 kDa started on day 3 of the last instar. The proteins were synthesized at a maximal rate on day 5 of the last instar, similarly as it was observed previously by Ray *et al.* [19]. It was also found that these proteins were rapidly secreted by the fat body and accumulated in the hemolymph (Fig. 3). The accumulation of [^{14}C]leucine-labelled proteins in the hemolymph started on day 3 of the last instar, which corresponds with the time of their synthesis in the fat body (Fig. 2).

We suggest that the proteins of 74, 76 and 81/82 kDa observed in our experiments represent the group of storage proteins of *Galleria*, described by Miller & Silhacek [14].

It is known that the development of *Galleria* larvae is inhibited by low temperature [10–12]. At 18°C *Galleria* larvae do not pupate and can persist for more than one year [10, 12].

In our studies a marked decrease of protein synthesis was observed in *Galleria* larvae kept at 18°C. This decrease was noted as early as after two days of exposure to low temperature. On the day 30, the rate of protein synthesis in the fat body was by about 70% below the initial rate of the first two days at 18°C. On the other hand, in the hemolymph of larvae reared at 18°C an accumulation of proteins of 74, 76 and 81/82 kDa was detected (Fig. 4A and B). It is interesting that the synthesis of these proteins in the fat body did not start simultaneously. The synthesis of 74 kDa and 76 kDa proteins started on day 2, and that of about 80 kDa on day 5 following the transfer of larvae from 30°C to 18°C. The synthesis of these proteins persisted

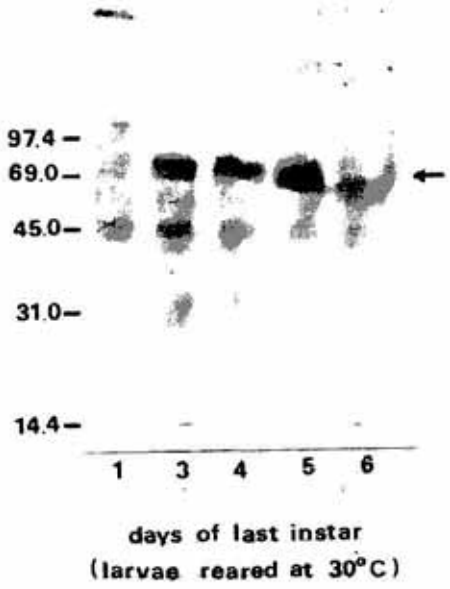

Fig. 2. Autoradiogram of proteins synthesized by the fat body of developing Galleria larvae.
The proteins were labelled *in vivo* (5 µCi of [^{14}C]leucine/larva). The fat body was taken from larvae (kept at 30°C) 3 h after injection of isotope. Proteins were separated on 10% polyacrylamide gel. The arrow marks the position of the group of proteins with molecular mass from about 74 kDa to 82 kDa.

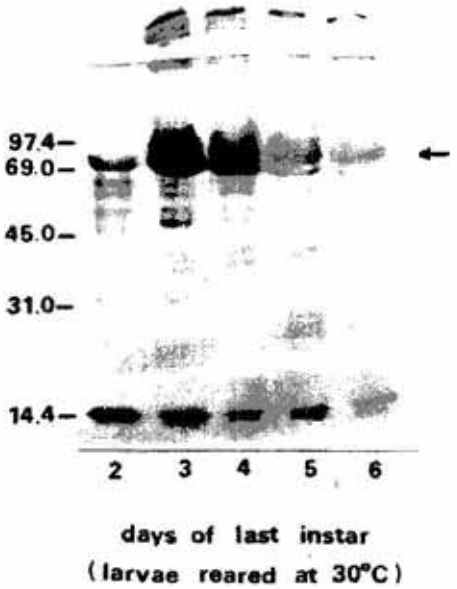

Fig. 3. Autoradiogram of proteins detected in the hemolymph of developing larvae.
The hemolymph was collected from larvae (kept at 30°C) 3 h after injection of isotope. The proteins were separated on 10% polyacrylamide gel. The arrow marks the proteins with molecular mass from 74 kDa to 82 kDa.

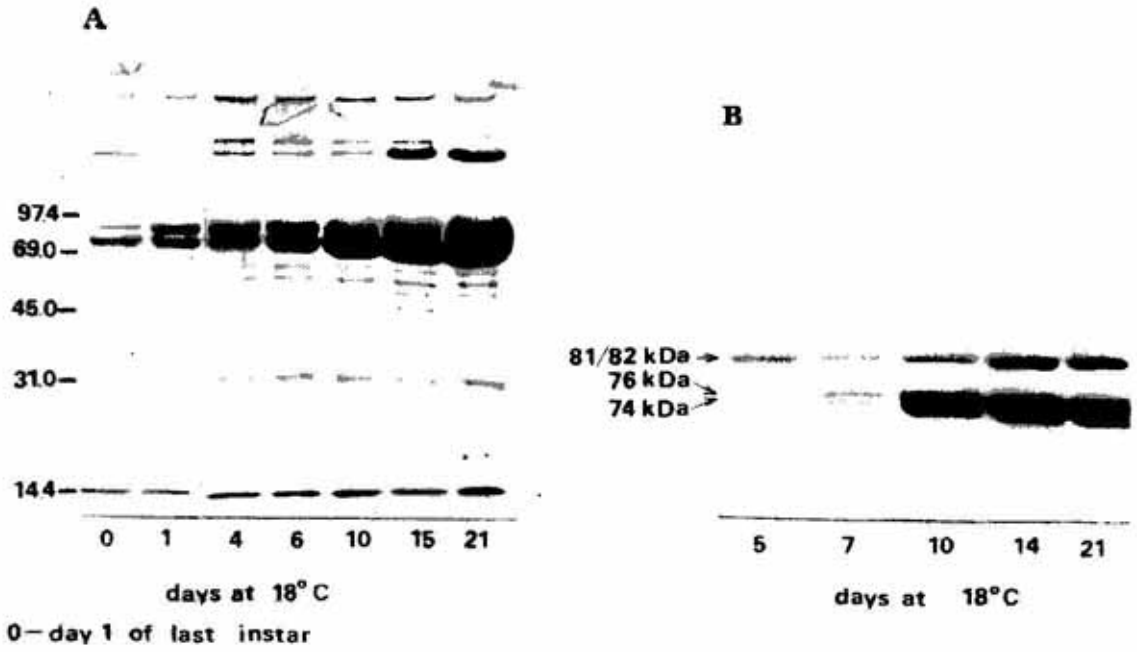

Fig. 4. Changes in the electrophoretic pattern of hemolymph proteins of larvae reared at 18°C.
One day old last instar larvae were transferred from optimal temperature (30°C) to 18°C, and then reared at this temperature for at least 4 weeks. Proteins were analyzed in 10% polyacrylamide gel (A) and in 7.5% polyacrylamide gel (B). Proteins in 0.5 µl of hemolymph were loaded into each slot of the gel.

at a high level until at least day 30 (Fig. 5). Newly synthesized proteins were secreted by the fat body and became detectable in the hemolymph on day 3 (Fig. 6).

Thus, our results indicate that the arrest of development of *Galleria* larvae is associated with the synthesis and accumulation of storage proteins.

A similar accumulation of storage proteins was also observed in diapausing adults of *Pyrrhocoris apterus* (L.) and of bean bug *Riptortus clavatus* [7, 8]. The function of these proteins in diapausing insects is still unclear. It was suggested that the storage proteins, constituting an amino-acid reservoir [7, 20], could be used as building material for post diapause morphogenesis and reproduction [1, 7, 8]. Some protein reserve is consumed during diapause [1]. This may also apply to *Galleria* larvae, the development of which is arrested by low temperature.

Presently, we are studying the influence of hormones on regulation of the induction, maintenance and termination of larval arrest in *Galleria*. It has been suggested that ecdysteroids are involved in these processes [12]. The titer of ecdysteroids in larvae kept at low temperature is very low, similar to that observed at the beginning of the last instar of developing larvae reared at 30°C [10].

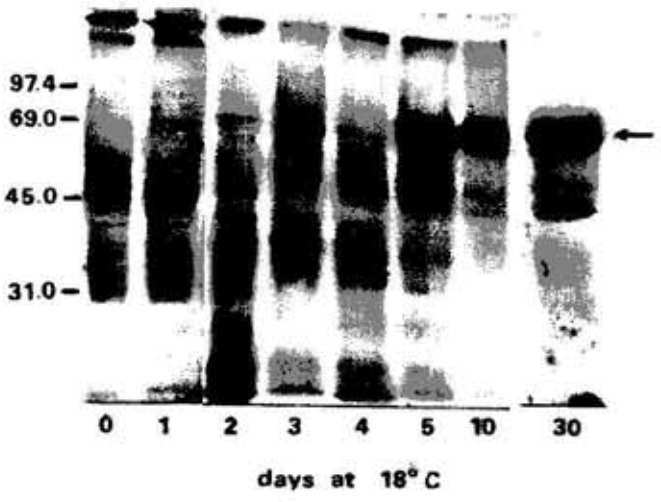

Fig. 5. Autoradiogram of [^{14}C]leucine labelled fat body proteins of larvae exposed to 18°C.
The proteins were labelled *in vivo* (5 µCi of [^{14}C]leucine/larva). The fat body was collected after 6 h of incubation with the isotope at 18°C. Proteins were separated by the electrophoresis on a 10% polyacrylamide gel. The arrow marks the position of the group of proteins with molecular mass from about 74 kDa to 82 kDa. 0, day 1 of last instar.

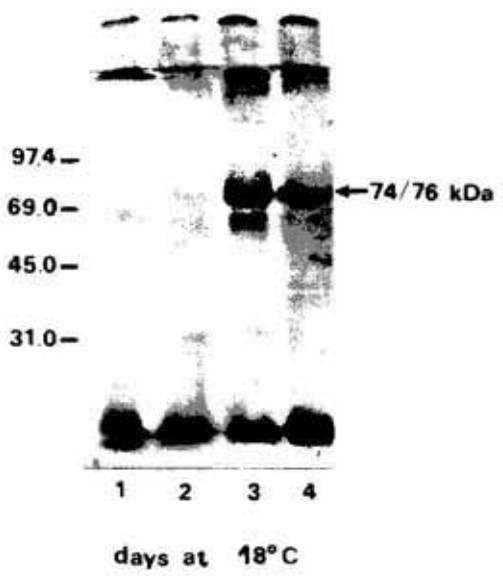

Fig. 6. Autoradiogram of proteins detected in the hemolymph of larvae exposed to 18°C.
Hemolymph was collected after 6 h of incubation with the isotope at 18°C. Proteins were separated on the 10% polyacrylamide gel in the presence of SDS. Equal amounts of radioactive material were loaded into each slot of gel.

In preliminary experiments we noted that exogenous 20-hydroxyecdysone applied *in vivo* at a dose of 5 μg/larva, inhibited synthesis of 74 kDa and 76 kDa proteins in the fat body of larvae exposed to low temperature. Ecdysteroids were also reported [21–23] to inhibit production of storage proteins in developing *Galleria* larvae.

Therefore, it appears that, ecdysteroids may be involved in regulation of the arrest of larval development in *Galleria mellonella*.

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