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The effect of cold shock on the immune response of the greater wax moth *Galleria mellonella* after infection with entomopathogenic bacteria *Bacillus thuringiensis*

Wpływ szoku zimna na odpowiedź immunologiczną barciaka większego *Galleria mellonella* po zakażeniu bakterią entomopatogenną *Bacillus thuringiensis*

SUMMARY

Insect immune system consists of only innate mechanisms relied on cellular and humoral branches. Many defence proteins and peptides exist or appear in response to infection in insect's hemolymph. The interaction between the infected host and the entomopathogen occurs in the conditions of external environment. In this work the greater wax moth larvae of *Galleria mellonella* were subjected to a temperature of 12^0C for a short period of time, directly before infection with entomopathogenic bacteria *Bacillus thuringiensis*. It appeared that the induction of the immune response was higher in cold-shocked animals than in larvae permanently reared at the optimal temperature of 28 ^{0}C. This enhanced immune response was manifested as higher antibacterial and lysozyme-type activity detected in full hemolymph, and as a higher level of peptides of molecular weight below 10 kDa having antibacterial activity. Moreover, other changes in the contents of proteins in the hemolymph were observed. These changes concerned *inter alia* apolipophorin III, the multifunctional protein of immune significance. Its level was higher in the hemolymph of animals pre-exposed to cold shock than in nonshocked, infected ones. Altogether our results indicate that the interdependence mechanisms occur between cold shock and the immune response.

Keywords: *Galleria mellonella, Bacillus thuringiensis*, insect immunity, cold shock

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STRESZCZENIE

Układ immunologiczny owadów składa się jedynie z mechanizmów wrodzonej odporności, w których skład wchodzi odpowiedź komórkowa oraz humoralna. Wiele białek i peptydów obronnych znajduje się na stałe lub pojawia się w hemolimfie w odpowiedzi na zakażenie. Interakcja między zainfekowanym owadem a patogenem odbywa się w warunkach środowiska zewnętrznego. W opisanych badaniach gąsienice mola woskowego *Galleria mellonella* poddawano krótkotrwałemu działaniu obniżonej temperatury tj. 12^0C bezpośrednio przed zakażeniem bakterią entomopatogenną *Bacillus thuringiensis*. Okazało się, że poziom odpowiedzi immunologicznej był wyższy u zwierząt poddanych stresowi zimna w porównaniu z zakażonymi owadami stale hodowanymi w temperaturze optymalnej, tj. 28^0C. Manifestowało się to zwiększoną aktywnością przeciwbakteryjną i aktywnością typu lizozymu pełnej hemolimfy oraz zwiększoną ilością indukowanych zakażeniem peptydów przeciwbakteryjnych o masie poniżej 10 kDa. Ponadto zaobserwowano inne zmiany w obrazie rozdzielonych elektroforetycznie białek hemolimfy. Dotyczyły one m.in. apolipoforyny III – wielofunkcyjnego białka zaangażowanego w mechanizmy odpornościowe owadów. Jego ilość była wyższa w hemolimfie owadów poddanych stresowi zimna w porównaniu z ilością w hemolimfie owadów niepoddanych działaniu stresu przed zakażeniem. Uzyskane wyniki wskazują na istnienie mechanizmów współzależności między szokiem zimna a odpowiedzią immunologiczną.

Słowa kluczowe: *Galleria mellonella*, *Bacillus thuringiensis*, odporność owadów, szok zimna

INTRODUCTION

Insects, like all multicellular organisms are exposed to a wide array of pathogens including bacteria, fungi and viruses. They are protected against infection by their anatomic barriers, for example chitin cover of the insect body, trachea, front and rear gut. Pathogens breaking this barriers cause induction of insect's immune response. It comprises of interconnected cellular and humoral branches (15). Cellular response involves phagocytosis, encapsulation and nodulation processes in which insect's blood cells, namely hemocytes, are engaged. This immediate reaction results in the elimination of foreign microorganism or, if not possible, sequestration thereof from the rest of the insect's body (22, 37). Humoral immune response engages *inter alia* the activation of phenol oxidase and the synthesis of defence peptides. The latter destroy infectious microorganisms mainly by destabilisation of their membranes. In the best understood insect model – *Drosophila melanogaster*, Toll and Imd pathways regulate the expression of genes encoding defence peptides. Both of them activate transcription factors homologous to mammalian NF-κB: Dif and Relish, respectively (12). Unlike vertebrates, insects are devoid of adaptive immune response based on lymphocytes and therefore they do not possess antibodies. Nevertheless, data coming from different laboratories underline the presence of a kind of immune memory which is based on the variability of receptors present on the surface of hemocytes and/or soluble in their hemolymph (11, 28). In the light of the fact that innate immune response is well evolutionary conserved, insects are good models to study its mechanisms as well as virulence factors of many human pathogens. While *Drosophila melanogaster* is broadly used for studying molecular aspects of insect immunity due to the availability of genetic tools, the greater wax moth *Galleria mellonella* is a good model to study biochemical aspects of the immune response, because the hemolymph, fat body and hemocytes of this insect can be easily obtained (8, 10, 17, 24, 29, 36). Some defence peptides as well as other immune relevant proteins have been identified in this organism (3, 5, 9, 16, 27, 32, 40). Some of them are synthesized by fat body and hemocytes and secreted to the hemolymph after infection and some of them are constitutively present in the hemolymph, although their level or activity may

change in immune-challenged animals. Among immune-relevant proteins constantly present in the hemolymph there are lysozyme and apolipophorin III (3, 21). Lysozyme is a protein of molecular weight of about 14 kDa of muramidase activity that hydrolyses β-1,4 linkage in the peptidoglycan layer of the bacterial cell (13). Apolipophorin III in turn is a multifunctional protein engaged in lipid transport to muscles during flight, regulation of apoptosis, and finally in many aspects of insect immune response (46).

The natural habitat of *Galleria mellonella* is beehives where it feeds on pollen, honey and bee wax. It causes disease called galleriose and this is the reason why it is considered as pest in agriculture. Within its natural environment this insect encounters various entomopathogens to which it has evolved immune response. One of them is a gram-positive bacteria *Bacillus thuringiensis*, which due to its entomopathogenic properties is used as a bioinsecticide (7). During the stationary phase of *B. thuringiensis* growth, a parasporal crystals are produced which contain Cry and Cyt toxins. In general, these toxins upon ingestion by susceptible insect, cause perforation of the gut allowing bacterial cells to reach hemocel, where they proliferate and cause septisemia (2, 31, 38). During the development of septisemia, *B. thuringiensis* secretes many virulence factors including phospholipases C, enterotoxins, hemolysins, cell surface proteins and metalloproteinases. Genes encoding these virulence factors are controlled by PlcR transcriptional activator (1, 20). Summarising, infection scenario may be described as a dramatic deadly fight between the host and the pathogen. While the host induces immune response to keep homeostasis, the intruder secretes virulence factors breaking the immune-relevant molecules of the infected host. This mutual interplay does not occur in the vacuum under the condition of external environment. Changes in the temperature for example may have an important effect on host-pathogen interaction. We reported before that exposing of *Galleria mellonella* larvae to elevated temperature directly before infection or rearing the infected animal at mild heat-shock temperature enhances its immune response (35, 42, 44). In this report we show that exposition of animals to cold shock before infection also has an influence on the defence response of the insects.

MATERIALS AND METHODS

Insects, bacteria and infection

The greater wax moth larvae of *Galleria mellonella* were reared on honeybee nest debris in darkness at 28^0C. In all experiments the last instar larvae were used. *Bacillus thuringiensis kurstaki* HD1 strain was obtained from Scientific Research Institute of Plant Protection Methods, Kishinev, Moldova. Bacteria were grown overnight in LB medium (1% bactotryptone, 1% NaCl, 0.5% yeast extract) at 37°C with shaking. Afterwards, the culture was diluted to the appropriate density of 4×10^3 in 5 µl. Bacteria were injected into larval hemocel with the use of Hamilton syringe. Injected larvae were reared at 28^0C.

Preparation of cell-free hemolymph and testing its total defence activity

The larvae were cooled down, surface sterilized and injured with a sterile needle. The hemolymph was sampled in Eppendorf tubes containing crystals of phenylthiourea to prevent melanisation. The obtained hemolymph samples were centrifuged (200 ×g, 5 min, 4°C) to sediment hemocytes and then the supernatant was centrifuged again (20,000× g, 15 min, 4°C) to get rid of all debris. The cell- free hemolymph was kept at -20°C until further use. The hemolymph collected from 8–10 larvae was used in each group.

The antibacterial activity of the cell-free hemolymph was determined by an inhibition zone assay. Plates containing *Escherichia coli* strain D31 (CGSC5165, Genetic Stock Centre, New Haven, CT) were prepared as described before (41, 42, 44). Five microliters of hemolymph of protein concentration 40 µg/ µl was applied on 2.7 mm holes made in solid medium and after

overnight incubation at 37 ^{0}C the growth inhibition zones were measured. The results were quantified according to Hultmark (14), cecropin B (Sigma, St. Louis MO) was used as a standard.

Lysozyme activity was assayed on agarose plates containing Soerensen buffer (0.066 M KH_2PO_4, 0.066 M Na_2HPO_4, pH 6.4) and freeze-dried *Micrococcus luteus* (Sigma). Its activity was measured as zone of digested *M. luteus* peptidoglycan and quantified according to Mohring and Messner (26). The activity was expressed as EWL (Egg White Lysozyme) equivalent (µg/ ml) (43).

Protein electrophoresis and immunoblotting

Hemolymph samples were loaded onto 12.5 % Tris-glycine polyacrylamide gel and separation was performed according to Laemmli (19). The gels were either stained with Coomasie Brilliant Blue or electroblotted onto Immobilon P membranes (Millipore). The membranes were then probed with antibodies developed in rabbits raised against *G. mellonella* apoLp-III (1: 2,500). The antibodies were a custom order obtained as described in (35, 45). As secondary antibodies goat anti-rabbit IgGs (1:30,000) conjugated with alkaline phosphatase were used. Immunoreactive bands were visualised by incubation with p-nitro blue tetrazolium chloride (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP). Quantitative analysis was performed using ChemiDoc MP system (Biorad).

Tris-Tricine SDS polyacrylamide gel electrophoresis

To get rid of high molecular weight proteins, methanol extracts of cell-free hemolymph were prepared as described before (44). After lyophilisation, the pellet was resolved in water and sample buffer was added. The samples were then loaded on 6.5% T, 6% C separating gel with "spacer" 10% T, 3% C and stacking gel 4% T, 3% C and after the run, the gel was stained with Coomasie Brilliant Blue. Details of Tris-tricine gel electrophoresis are described in Schagger and von Jagow (30).

Bioautography

Polypeptide antibacterial activity *in situ* after SDS gel electrophoresis was detected as already described (44). Shortly, hemolymph proteins were resolved on 13.8% gel and electrophoresis was performed as described above. After protein renaturation, the gel was overlaid with *E. coli* D31 in nutrient agar containing lysozyme (2.5 mg/ ml) to increase the sensitivity of the method. After incubation at 37^{0}C, the growth inhibition zones were observed. Samples containing 100 µg proteins were applied on the gel. For gel image acquisition, documentation and quantification, a video image analyser Chemi Doc MP ImagingSystem (Bio Rad) was used.

RESULTS

Infected *Galleria mellonella* pre-exposed to cold shock shows enhanced defence activity

In order to test the effect of short-term exposition of *G. mellonella* larvae to cold-shock on its ability to induce immune response, the last instar larvae were placed to the temperature of 12^{0}C for 15, 30 and 60 min. Afterwards, they were injected with *B. thuringiensis* and further reared at optimal temperature of 28^{0}C for 5 hours. Other groups of animals were not subjected to cold-shock before infection. Hemolymph collected from each group of larvae (obtained as described

in Materials and Methods section) was tested for antibacterial and lysozyme activity. As Fig. 1A shows, injection of bacteria into larval hemocel caused induction of the insect immune system. In larvae pre-exposed to cold shock, at all time of exposition (15, 30 and 60 min.) this detected activity was from 1.3 to 2.2 times higher in comparison to non cold-shocked animals, five hours after infection. In case of lysozyme, the highest activity was observed in the hemolymph of larvae exposed to cold-shock for 60 min, which was almost twice higher than in non-shocked, infected larvae (Fig 1 B).

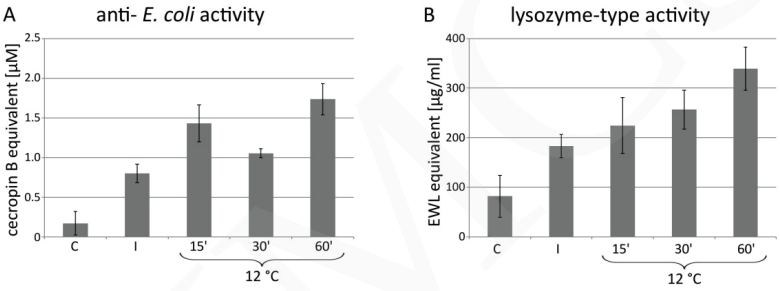

Fig. 1. Antibacterial (A) and lysozyme- type (B) activity in the hemolymph of *G. mellonella* larvae. The respective activity in cell-free hemolymph was assayed as described in Materials and Methods section. The larvae were infected with *B. thuringiensis* (4×10^3 cells) and placed to 28^0C. The hemolymph was collected 5 hours after infection (I). Some larvae, directly before infection were exposed to cold-shock by placing them to 12 ^{0}C for 15, 30 and 60 min. as indicated. The results are a mean from 3 assays +/- SD. The hemolymph activity in non-infected larvae was assayed as a control (c).

Protein pattern of *G. mellonella* hemolymph exposed or nonexposed to cold shock is affected after infection

We compared protein patterns of *G. mellonella* hemolymph. Total hemolymph proteins were subjected to 12.5% SDS polyacrylamide gel. As can be seen in Fig. 2A in animals pre-exposed to cold-shock for 30 and 60 min the amount of polypeptides present in the gel in the position between 97.4 and 66.2 kDa marker proteins (arrow a) was slightly lower in comparison to infected larvae not subjected to cold shock. Their molecular mass corresponds to lipophorins. Also, at the same slots, the amount of other polypeptides, described as b and c was lower in pre-shocked, infected animals in comparison to infected larvae non-exposed to cold shock.

We also compared the pattern of low molecular weight proteins in all hemolymph samples. In order to get rid of high molecular weight proteins, methanol extracts were prepared as described in Materials and Methods section and extracted proteins were resolved with the use of Tris-tricine electrophoresis. We noticed higher amount of proteins in the hemolymph of infected animals pre-exposed to

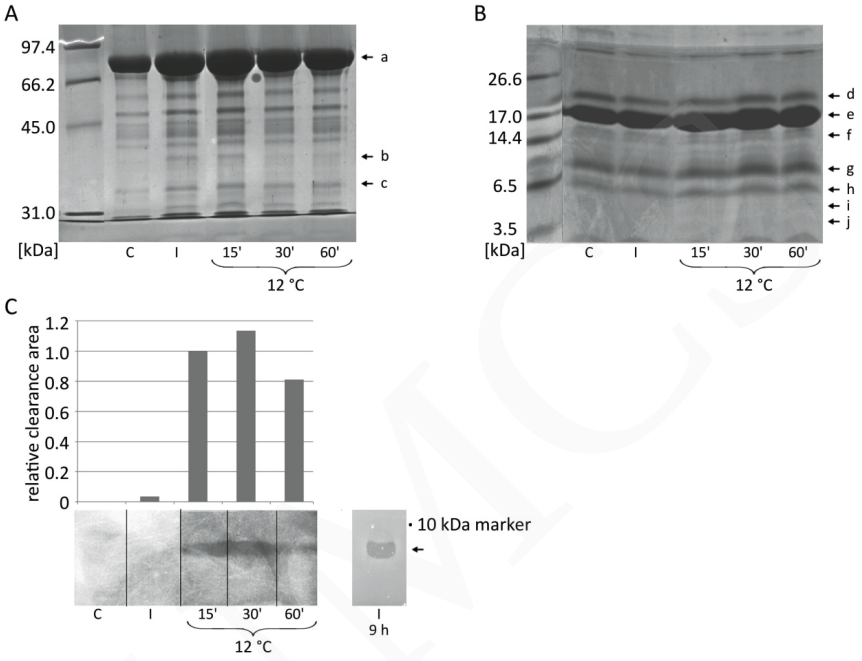

Fig. 2. The protein pattern of *G. mellonella* hemolymph after separation on Tris-glycine (A) or Tris-tricine (B) gels and detection of antibacterial activity in separated hemolymph proteins (C).

The hemolymph was collected from larvae not-exposed (I) or pre-exposed to cold shock for 15, 30 and 60 minutes as indicated, directly before injection of *B. thuringiensis* cells (4×10^3) and from naive larvae (c). After infection larvae were reared for 5 hours at 28 ^{0}C. Additionally in (C), one group of nonshocked, infected larvae were reared after infection for 9 hours at 28 ^{0}C, as indicated.

In A, 10 µg of cell-free hemolymph proteins were loaded on the gel. In B, methanol extracts from cell-free hemolymph were prepared and twenty micrograms of proteins were loaded on the gel for staining. In C, 100 µg of hemolymph proteins were loaded and bioautography was performed as described in Materials and Methods section. The growth inhibition zones reflect the activity of antimicrobial peptides. Bars show the quantization of peptide activity as relative clearance zones in relation to zone observed in infected animals exposed for 15 min to cold shock (third slot).

cold shock in comparison to their amount in non-shocked, infected ones (described as d to j in Fig. 2B). Molecular weight of protein of 18 kDa (designed e) corresponds to apolipophorin III (apoLp-III), which is intensively extracted from the hemolymph. Furthermore, the molecular weight of protein (designed as f and h) corresponds to the mass of lysozyme and anionic peptide-2, respectively (21). Both mentioned proteins are known to be constitutively present in *Galleria* hemolymph.

Finally, the molecular mass of peptides below 6.5 kDa (i and j) corresponds to weight of infection-induced *G. mellonella* defence peptides. In the next step we tested the antibacterial activity of hemolymph proteins separated in the gel. After

the run and the renaturation of separated proteins (described in detail in Bioautography chapter in Materials and Methods section) the gel was overlaid with agar medium containing living *E. coli* cells and incubated at 37 ^{0}C. As shown (Fig. 2C) the inhibition growth was observed below 10 kDa marker protein (indicated by arrows). Five hours after infection the inhibition zone was not visible in infected larvae non-exposed to cold shock. Although 9 hours after infection, when the infection was more developed, the in-gel activity of defence peptides in unshocked, infected larvae was clearly visible (right part). In case of infected larvae pre-exposed to cold shock, the in gel activity of separated defence peptides was visible already 5 hours after infection. This confirmed our observation concerning total activity of *G. mellonella* hemolymph presented above.

The effect of cold-shock on the apolipophorin III level in the hemolymph of infected *G. mellonella* larvae

The results presented in Fig. 2B concerning the enhanced level of 18 kDa protein in the hemolymph of infected larvae pre-exposed to cold shock prompted us to follow the level of apoLp-III with the use of immunodetection technique. The obtained results confirmed our observation on stained gel (Fig. 3).

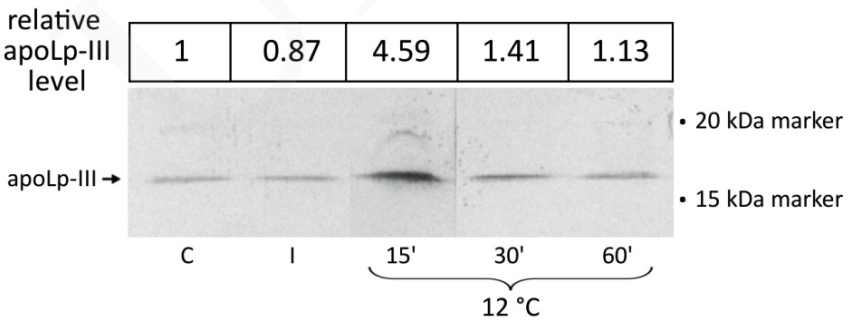

Fig. 3. The level of apoLp- III in the hemolymph of *G. mellonella* larvae. The hemolymph was collected 5 hours after injection of *B. thuringiensis* cells into larval hemocel (I). Some larvae directly before injection were exposed to 12 ^{0}C for 15, 30 and 60 min. as indicated. Samples containing 3 μg of total proteins were resolved by Tris-glycine SDS-PAGE, electroblotted onto Immobilon membranes, and probed with anti-apoLp-III antibodies. Apolipophorin-III is indicated by an arrow. Densitometric analysis in relation to the amount of apoLp-III in the hemolymph of non-infected larvae (control, c) is shown in the table above.

Consistent with our results presented before, infection caused slight decrease in the amount of apoLp-III detected five hours after *B. thuringiensis* injection (35). Comparing its amount in nonshocked and cold-shocked infected animals, the level of this 18 kDa protein was significantly higher in the latter ones. The

higher amount of apoLp-III was observed in infected animals pre-shocked for 15 min. and it was lower when larvae were exposed to cold-shock for longer period of time (30 and 60 min.), remaining at higher level than in infected, non-shocked animals.

DISCUSSION

Insects are remarkably resistant to microbial infection. Despite the absence of adaptive immunity they have developed effective mechanisms of recognition and elimination infectious agents. During their life, they are exposed not only to infection but also to many abiotic stresses such as changing the temperature, osmotic and oxidative stress, desiccation etc. Sometimes they encounter a few kinds of stresses at the same time or directly one after another. In insects like in other ectothermic animals, which are seasonally exposed to non-optimal temperature, it's changes affect metabolic activities, development and growth (33). Cold exposure cause an increase in the proportion of unsaturated and saturated acids in biological membranes, up-regulation of a number of genes encoding stress proteins and antifreeze proteins, accumulation of sugars and polyols. Interestingly, experience of one kind of stress may cause an increase in tolerance to another one and this phenomenon is called adaptive cross-tolerance (34). Also in the literature the term hormesis is used describing similar phenomenon. It consists of the fact that a negative for the optimal growth input which does not induce severe damage, can enhance the ability of the organism to respond more effectively to other stress (23).

In this report we show that short-term exposure of *Galleria mellonella* larvae to mild cold-shock temperature effects its ability to induce the immune response. Exposition of animals to 12 ^{0}C for 15, 30 and 60 min. directly before infection with *B. thuringiensis* enhanced the antibacterial activity appearing in the hemolymph in response to infection in comparison to non-shocked larvae. It is worth to mention that long-term exposition of *G. mellonella* larvae to sub-optimal temperature such as 18^{0}C results in developmental arrest of larvae and cause a marked delay in pupation, which is described as facultative larval diapause (4, 25). Also the protein synthesis has been shown to be affected (18). Despite the fact that 12^{0}C is stressful for the wax moth, short-term exposure to this conditions appeared to be beneficial for infection-induced appearance of defence activity in larval hemolymph. Also, the activity of lysozyme appeared to be higher in pre-shocked animals. However in this case the longest cold-treatment of animals appeared to be the more beneficial. As mentioned in the Introduction, lysozyme is constantly present in *G. mellonella* hemolymph and, as we presented here and before, its activity increases in response to infection. This 14 kDa protein destroys bacterial peptydoglycan and was shown to act in synergy with antibacterial peptides

against bacterial infection. Also its non-enzymatic antifungal activity is underlined (6, 39).

In agreement with total defence activity detected in whole hemolymph, an analysis of protein patterns showed differences in some polypeptides. The amount of protein of 14 kDa was observed to be higher in shocked animals in comparison with infected non-shocked ones, which correlates with lysozyme activity detected. Moreover, in cold-shocked larvae the amount of proteins of molecular weight below 10 kDa was higher in comparison with infected, non-shocked *G. mellonella* larvae. The assumptions that they could be antimicrobial peptides were confirmed by bioautography technique. Inhibition zones were observed only in infected animals in the position below 10 kDa marker. While after 5 hours post-infection they were not detectable in non-stressed larvae, in animals exposed to cold-shock they were clearly visible and the widest were in animals pre-exposed to 12^0C for 30 min.

Finally, the differences in the level of apoLp-III were observed. Similarly like we reported before (35) its level slightly decreased 5 hours after infection but in cold-shocked animals it was significantly higher. Apolipophorin III may exist in the hemolymph in lipid-free and lipid-bound state due to its role in lipid transport. As a part of defence system this protein stimulates defence activity of peptides, activates prophenolooxidase cascades and finally has an antimicrobial activity itself. Also it acts as Pathogen Recognition Receptor due to its ability to bind to components of bacterial and fungal cell wall such as LPS, β-1,3-glucan and lipoteichoic acids (LTA). Summarising, it is a protein involved in different biochemical processes so its level in the hemolymph is a trade-off between many aspects of insect physiology. Its increased amount, together with enhanced level of inducible defence peptides and lysozyme may explain the higher antibacterial activity appearing in the hemolymph in response to infection in cold-shocked animals in comparison with non-shocked ones. In this place it is worth to recall our results showing that exposition of *G. mellonella* larvae to elevated temperature enhanced its immune response and has an influence on host-pathogen interaction (35, 42, 44). Heat shock and cold shock have many common mechanisms. For example, both induce the expression of so-called heat shock proteins (more adequately – stress proteins). In the light of these results it seems that temperature stress fits perfectly to the phenomenon of cross-protection, meaning that the popular saying "what does not kill you makes you stronger" has its biochemical justification.

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