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SHORT COMMUNICATION

Immune priming of *Galleria mellonella* larvae with *Bacillus thuringiensis* affects coagulation and phenoloxidase activity upon subsequent infection

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Abstract

Immune priming is a phenomenon that allows invertebrates, which are devoid of acquired immunity, relying on memory T-cells and antibodies, to achieve better protection after subsequent infection. In this short report, we present new investigations of the immune response of primed *Galleria mellonella* larvae after infection with *Bacillus thuringiensis*. We compared two related aspects of immune response – hemolymph coagulation and the activity of phenoloxidase in the primed and non-primed larvae after the injection of the lethal dose of *B. thuringiensis*. The *in vivo* assay showed that coagulation of hemolymph in response to the bacterial injection occurred more efficiently in the primed animals in comparison to the non-primed ones. Further, we showed that the activity of phenoloxidase was also higher in the primed, infected larvae. Both parameters of insect immune response may contribute to the increased resistance of primed *G. mellonella* to further infection with *B. thuringiensis*.

Key Words: greater wax moth; hemolymph coagulation; insect immune memory; insect immunity; melanisation

Introduction

Insects possess only innate immune mechanisms to fight infections (Buchmann, 2014). The cellular branch of their defence system involves hemocytes and comprises phagocytosis or entrapping intruders in structures called nodules and capsules (Falabella *et al.*, 2012). The humoral branch is based on soluble factors and involves *inter alia*: synthesis of antimicrobial peptides and activity of enzymes such as phenoloxidase and transglutaminase. Antimicrobial peptides directly destroy invading microorganisms mainly by perforation of their membranes (Hillyer, 2016). Phenoloxidase and transglutaminase are involved in the synthesis of melanin and clot formation, respectively. In principle both melanization and coagulation require coordinated activity of the humoral and cellular defence components. Both processes are triggered in response to wounding and infection and serve as the basis for subsequent interaction of host's molecules with intruding microbial cells (Wojda and Vertyporokh, 2017). During coagulation, soluble hemolymph components are converted into an insoluble clot. In this process, insect hemocytes degranulate, releasing microparticles of reversed membrane polarity, exposing negatively charged lipids from the inner leaflet of the membrane bilayer. This attracts other hemocytes and all components necessary for coagulation. In many insect species lipophorins including apolipophorin III serve as a protocoagulant (Theopold *et al.*, 2002). A protocoagulant is a substrate for transglutaminase, i.e. an enzyme catalysing the formation of isopeptidic bonds converting coagulogen into insoluble coagulin. The exact mechanism of insect coagulation is unravelled. Li *et al.* (2002) identified proteins involved in hemolymph coagulation in *G. mellonella*. Besides the known members of coagulation system, like lipophorins, they found components of a prophenol-activating cascade, supporting the idea that both coagulation and melanization systems work together during clot formation. Haine *et al.* (2007) showed that hemolymph clotting in insects is

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enhanced when associated with non-self and is an important feature of immune response. It localises immune effectors near injury and creates compartmentalisation of the open hemocel, preventing intruder invasion. The phenoloxidase system is activated after injury or infection and is involved in synthesis and deposition of a brown-black pigment called melanin, occurring at the site of injury or infection (Krautz *et al*., 2014). This process is catalysed by phenoloxidase (PO), a copper-containing enzyme oxidising phenols to quinones, which further undergo polymerisation and form insoluble melanin. In physiological conditions, this enzyme exists as an inactive precursor called prophenoloxidase (proPO), which is released by hemocytes (oenocytoids in *G. mellonella*) upon immune challenge and activated by limited proteolysis by a cascade of calcium-dependent serine proteases (Kanost *et al*., 2004). Melanin and intermediate products such as quinones and free radicals are highly toxic to invading microorganisms. The process is tightly controlled by serine protease inhibitors – serpins, which are released simultaneously with other components of the proPO system (Cerenius *et al*., 2008). The need of control arises due to the toxicity of free radicals and other intermediate products to host tissues as well. Prophenoloxidase from *G. mellonella* was purified and its mass was established at 66.2 kDa (Kopacek *et al*., 1995; Demir *et al*., 2012).

The model insect used in this study is the greater wax moth *Galleria mellonella.* In nature it lives in beehives, or, more often, in slices of stored wax, feeding with wax and pollen (Wojda, 2017). It can be infected *via* the oral route with its natural pathogen *Bacillus thuringiensis*. The toxins of this bacteria (Cry and Cyt) cause gut perforation, thereby allowing bacteria to reach the hemocel and colonise the insect body. Injured cuticle is another gate of infection. Both infection routes lead to the presence of bacteria in the hemocel and to the development of septicaemia, which is a cause of *G. mellonella* death (Wojda, 2017).

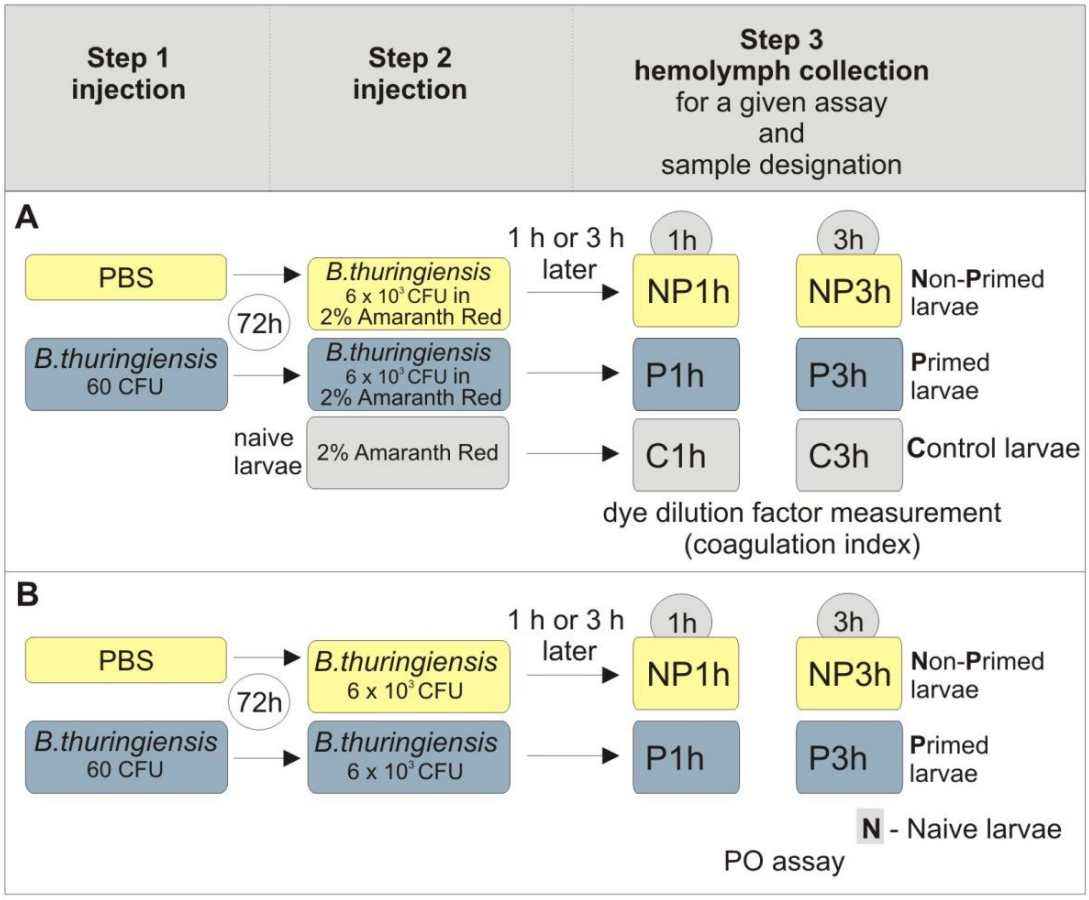

Fig. 1 The scheme presenting the design of experiments concerning coagulation (A) and PO assay (B). In both types of experiments, one group of larvae was injected with PBS (non-primed larvae, NP), while another one with the low dose of *B. thuringiensis* (60 CFU/larvae, primed larvae, P). After 72 hours, both groups were injected with the lethal dose of *B. thuringiensis* (6×10^3 CFU/larvae; infected larvae). In case of coagulation assay, the amaranth red was added to the injection mixture to the final concentration of 2%. Consequently the naive larvae were injected with 2% amaranth red only (control, C). Larvae were left at 28 ˚C for 1 and 3 hours. Then, the hemolymph was collected for checking dilution factor and for PO assay. The hemolymph from the naive larvae (N) was also collected for PO assay

There is an increasing number of reports regarding insect immune priming (for the review see Cooper and Eleftherianos, 2017). This term concerns increased resistance upon subsequent infection with the same or another pathogen. The regulatory mechanism of this phenomenon is unknown but attracts considerable attention because it reveals that, despite possessing only the innate immune mechanism, the insect can somehow "remember" previous infection and respond more effectively upon re-encountering of a given pathogen (Chambers and Schneider, 2012).

We have published before that *G. mellonella* larvae primed with a low dose of *B. thuringiensis* were more resistant for further infection with a high dose of the same bacteria in comparison to the non-primed ones (Taszłow *et al*., 2017). We have already shown that increased resistance of *G. mellonella* larvae pre-exposed to a low dose of *B. thuringiensis* correlated with enhanced hemolymph activity without stronger immune-related gene expression, suggesting differences in the protein turnover in the infected larvae (Taszłow *et al*., 2017). In this short report we continue this research, presenting that priming affects also coagulation process and phenoloxidase activity in the hemolymph of *G. mellonella,* which may also contribute to increased resistance of the primed larvae to further infection.

Materials and methods

Insects and infection

The larvae of *Galleria mellonella* (Lepidoptera: Pyralidae) come from breeding conducted in the Department of Immunobiology, Maria Curie-Sklodowska University, Lublin. The larvae were reared on honeybee nest debris in darkness at 28 °C. To infect them, the last instar larvae of 200 mg weight were injected in the last proleg with the indicated number of vegetative *Bacillus thuringiensis* cells (*B. thuringiensis kurstaki* HD1, *Bacillus* Genetic Stock Centre, The Ohio State University, Department of Biochemistry) in PBS buffer (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4, pH 7.4). Bacteria to be injected were grown in LB medium (1% bactotryptone, 1% NaCl, 0.5% yeast extract) at 37 °C until OD_{600} reached about 1.0; next, they were centrifuged at 8,500×g and suspended in PBS buffer to appropriate density. The number of bacteria was estimated based on OD_{600} and CFU (colony forming units) counting. 6×10^1 and 6×10^3 bacterial CFU in PBS buffer, in the volume of 5 µl were injected into each larvae for priming and for infection, respectively.

Bleeding the larvae and preparation of cell free hemolymph

To obtain hemolymph, the larvae were anaesthetised in ice-cold water, ethanol sterilised and injured with a sterile needle. Hemolymph was collected to Eppendorf tubes kept at 4 °C and containing a few crystals of phenylthiourea to prevent melanization. It was then centrifuged at 200×g for 5 min to pellet hemocytes, and then at 20,000×g for 10 min at 4 °C. The cell-free hemolymph was kept at -20 °C if necessary.

Experiment design and hemolymph in vivo coagulation assay

For our needs, we developed an *in vivo* coagulation assay based on the assay published by Haine *et al*. (2007) with modifications. The coagulation index was measured as the dilution factor of an amaranth red dye injected into the larval hemocel. In this method, the dye is incorporated into the coagulating hemolymph. The more dye is incorporated, the less it will diffuse through the open hemocel and the hemolymph obtained will have less intensive colour. The amount of the dye in the hemolymph obtained from individuals is compared to the *in vitro* dilution factor of amaranth red. To prepare a calibration curve, a serial dilution of 2% amaranth red was prepared and 5 µl of each dilution was added to 195 µl of the Ringer solution (172 mM KCl, 68 mM NaCl, 5 mM $NaHCO_3$, pH 6.1) in a 96-well microplate. The absorbance was measured at 520 nm. Then, the calibration curve was made based on the obtained OD *versus* the dilution factor of amaranth red.

To check *in vivo* coagulation in the infected, non-primed and primed larvae, one group of the larvae was primed with 60 CFU of *B. thuringiensis* / larvae in PBS buffer while another one was injected with PBS only. The priming dose (6×10^1 CFU) was chosen on the basis on the preliminary experiments analysing the effect of different bacterial doses on the viability of *G. mellonella* larvae. Because all injected doses appeared to produce some mortality, we have chosen very low number of CFU for the injection. Approximately 70% of larvae injected with this dose survived, as we reported before (Taszłow *et al*., 2017).

Both groups (primed and non-primed) were left at 28 °C for 72 hours, and then infected with 6×10^3 *B. thuringiensis* in 2% amaranth red. As a control, 5 µl of 2% amaranth red alone was injected into naive larvae. The detailed scheme of the experiment is presented in the Fig. 1A. One and 3 hours after the second injection, the hemolymph from each larva was collected individually after injury near the head. The hemocytes were sedimented as described above. Five microliters of cell-free hemolymph from each larva were added to 195 µl Ringer solution to measure the absorbance. The measurement was performed in triplicate for each larva and the average value was taken. The obtained values were re-calculated into the coagulation index based on the prepared calibration curve.

Experiment design and phenoloxidase activity assay

For phenoloxidase assay the priming was performed as described above for coagulation assay. After 72 hours both groups (primed and non-primed) were challenged with 6×10^3 CFU of *B. thuringiensis* in PBS and the hemolymph was collected 1 and 3 hours later for phenoloxidase assay. The detailed scheme of workflow is presented in the Fig. 1B. The hemolymph from 10 larvae of each group was collected in every independent experiment and the cell-free hemolymph was obtained as described above. The phenoloxidase activity in such prepared hemolymph was assayed.

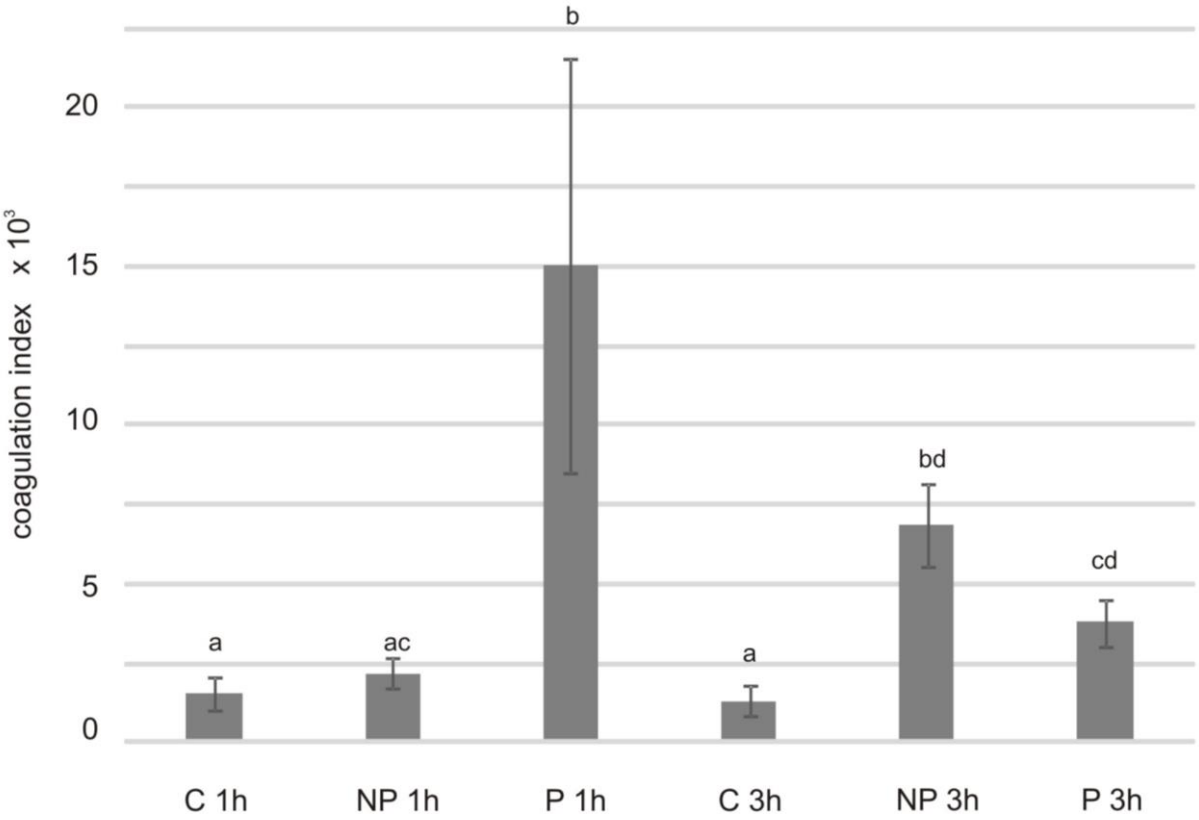

Fig. 2 Coagulation index in the primed (P) and non-primed (NP) larvae at the indicated time-points after injection with the lethal dose of *B. thuringiensis*; (C) - control larvae (amaranth red injected to naive larvae). The experiment was performed as shown in Fig.1A. Two independent experiments were performed in which the coagulation was assayed individually for each larva on the total number of 14-16 larvae in each group (+/- SD, standard deviation). Values with different letters are significantly different ($p < 0.05$; Kruskal-Wallis One Way ANOVA on Ranks, Tukey's post-hoc test)

PO catalyses melanin synthesis from the coreless substrate L-DOPA (L-3,4-dihydroxyphenylalanine). Synthesised melanin causes an increase in the absorbance values. Cell-free hemolymph containing 80 μg of protein in the volume of 2 μl was added to 16 μl of buffer A (50 mM Tris-HCl, 150 mM NaCl, 5 mM $CaCl_2$) in a 96-well plate and left for 20 min at room temperature. Afterwards, 180 μl of 2 mM L-DOPA in buffer B (50 mM Na_2HPO_4, 50 mM NaH_2PO_4) were added and the absorbance at 490 nm was measured immediately (time 0) in relation to a sample containing water instead of hemolymph and after specified time-points after substrate addition: 5, 10, 15, 20, 30, 45, 60, 75, 90, and 120 min. The protein concentration was estimated using the Bradford method (Bradford, 1976).

Statistical analysis

For statistical analysis, Sigma Stat 4.0 and Statistica 13 software were used. Kruskal-Wallis One Way ANOVA on Ranks, Tukey's post-hoc test, and Student t-test for depended samples were used. Normality of data was checked with the Shapiro-Wilk test. The differences were regarded as significant at $p < 0.05$.

Results and Discussion

We have found differences in both coagulation and phenoloxidase activity in the primed and non-primed *G. mellonella* larvae after the infection with *B. thuringiensis*, which may contribute to the differences in their susceptibility to these bacteria reported before (Taszłow *et al.*, 2017). The coagulation index in the primed larvae was five-fold higher than in the non-primed ones 1 hour after infection but not at 3 hours time point (Fig. 2). This means that the coagulation process in the primed larvae is faster and more efficient than in the non-primed ones. As mentioned in the Introduction, hemolymph can coagulate on the surface of infecting microorganisms or groups of microorganisms. Coagulation serves as one of the first immediate reactions to infection (Theopold *et al.*, 2002; Haine *et al.*, 2007). By the time the transduction pathways are activated and synthesis of antimicrobial peptides occurs, quick coagulation may prevent or rather limit proliferation of an intruder (Hillyer, 2016). It is worth to mention here, that insect circulatory system is open and therefore insects are not in danger of thrombosis (Trenczek *et al.*, 1988). That is why the coagulation of hemolymph

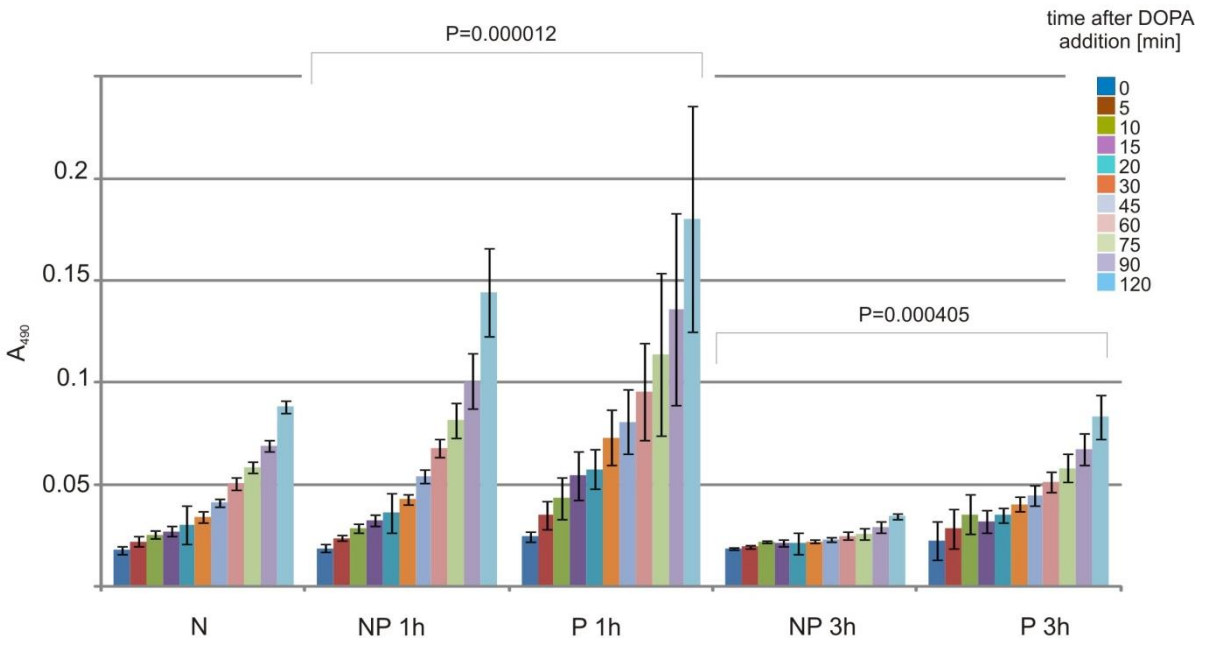

Fig. 3 PO activity measured in the primed (P), and non-primed (NP) *G. mellonella* larvae at the indicated time-points after infection with the lethal dose of *B. thuringiensis* (+/- SD, standard deviation); N – naive larvae. Three independent experiments were performed. In each experiment, hemolymph from ten larvae was pooled. Student t-test for depended data showed significant differences between values in the primed and non-primed larvae both 1 and 3 hours after infection (P<0.05; exact P values are given in the figure)

in insects is more important for innate immune defence than blood coagulation in mammals, the latter one mainly preventing blood efflux. Furthermore, micro cloths around the intruder, apart from separating it from the rest of the body, may serve as a danger signal participating in switching on the immune response (Ming *et al*., 2014).

The PO activity was higher in the primed larvae in comparison to the non-primed both 1 and 3 hours after the infection (Fig. 3). Probably, like it was in case of coagulation, the activation of PO is quicker in the primed larvae in comparison to the non-primed. It is worth to mention here, that at the later time points after infection, i.e. 7, 9 and 11 hours, there were no differences in the activity of PO between the primed and non-primed groups of larvae (Taszłow and Wojda, data not shown). We noticed that 3 hours after infection the activity of PO was lower than at one hour, respectively in the non-primed and primed larvae (P=0.00842 for the non-primed and P=0.002 for the primed; not shown in the figure). It is known that *B. thuringiensis* secretes some products as virulence factors that may inhibit activation of this enzyme.

Higher phenoloxidase activity in primed animals is likely to have an influence on their condition by affecting both cellular and/or humoral aspects of insect immunity. This enzyme in non-challenged larvae is present in hemolymph at a very low level but after injury or in response to infection, it is released from oenocytoids with simultaneous activation of zymogen into an active enzyme (Bidla *et al*., 2009). Synthesized melanin deposited on the surface of intruder may allow its faster phagocytosis. Also, nodulation could be affected. As mentioned in the Introduction, melanin can be deposited around the pathogen(s) during nodule formation and intermediate products releasing during melanin formation are highly toxic to pathogens closed in such structures (Falabella *et al*., 2012). Additionally, the fact that the defence molecules in the primed animals stay active longer in the hemolymph (Taszłow *et al*., 2017), enhanced PO activity may somehow influence the turnover of defence molecules, i.e. a process that is totally unknown in insects.

Considering the results presented here, the question may arise whether higher bacterial dose used for priming would enhance the coagulation and melanisation processes upon recurring infection. However, it is difficult to check, due to the very high pathogenicity of *B. thuringiensis*. On the other hand, the high pathogenicity may explain the fact that priming effect can be achieved by relatively low number of bacteria. It seems that even 60 CFU inside the hemocel may pose a significant immune challenge necessary to "leave an imprint" on the insect immune system to give a better protection against recurring infection.

Summarising, we have found here that two aspects of *G. mellonella* immunity are affected by priming: hemolymph coagulation and PO activity, which may contribute to the higher resistance of the primed larvae to re-infection. The question remains about the mechanism regulating this phenomenon.

In other words, how did the larvae "learn the lesson" from the first encountering of a pathogen during priming? Further studies are needed to understand the way the innate immunity can be modulated to protect insects against repeated infections. The discovery of the improved immune response in invertebrates have permanently switched our classical thinking of the features of innate and acquired mechanisms of defence. A fascinating answer may be provided by further studies on insect "immune memory".

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