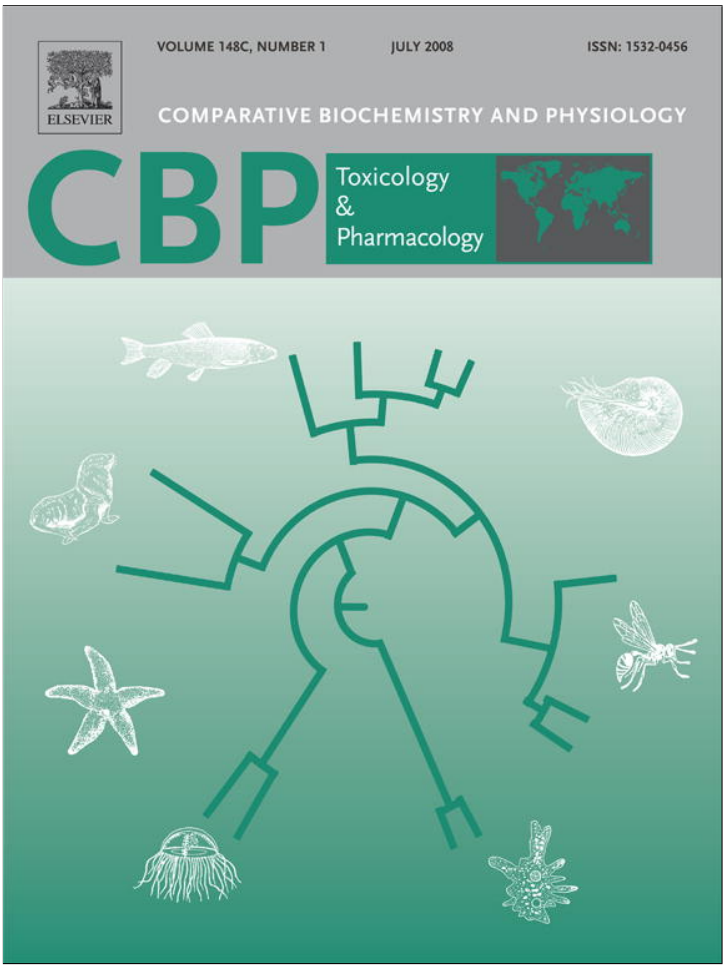

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# Effect of bacterial infection on antioxidant activity and lipid peroxidation in the midgut of *Galleria mellonella* L. larvae (Lepidoptera, Pyralidae)

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ABSTRACT

*Bacillus thuringiensis* is one of the most widely used sources of biorational pesticides, as well as a key source of genes for transgenic expression to provide pest resistance in plants. In this study the effect of *Bacillus thuringiensis ssp. galleriae* (Bt) infection on the activity of superoxide dismutase (SOD), glutathione *S*-transferase (GST), catalase (CAT), concentrations of oxidated and reduced thiols (RSSR/RSH) and malondialdehyde (MDA) was tested in the midgut of *Galleria mellonella* larvae. We found that Bt infection resulted in increased activities of SOD, GST, malondialdehyde and RSSR/RSH ratio the first day after inoculation. However, catalase activity decreased on the first and following days after bacterial infection by Bt. Our results confirm the hypothesis that Bt infection increases the level of oxidative stress in the larval midgut. In light of this study, it seems possible that oxidative damage contributes to cell death in the midgut during bacteriosis.



## 1. Introduction

*Bacillus thuringiensis* (Bt) is a gram-positive entomopathogenic bacterium that is active against more than 150 pest species, including the greater wax moth *Galleria mellonella* L. (Gill et al., 1992; Bravo et al., 2005). *G. mellonella* is a harmful pest of beehives, the larvae of which tunnel through the combs feeding on pollen, wax and honey. The toxicity of *B. thuringiensis* is due to the production of crystalline protein protoxins, known as δ-endotoxins (Obukowitz et al., 1986; Lampel et al., 1994; Broderick et al., 2006). Solubilized protoxins are activated by midgut proteases and bounded with the receptors of the epithelial cells (Pigott and Ellar, 2007). The toxins insert themselves into the cell, where they form pores that lead to cell lysis, subsequently causing insect death (de Maagd et al., 2003; Bravo et al., 2005). However, alternative models of insect death from Bt, involving cellular signaling leading to cell death, have also been suggested (Zhang et al., 2005; 2006; 2008; Higuchi et al., 2007). It is well known that the main forces of cell lysis under intestinal pathogenesis of vertebrates are lipid peroxidation and the generation of reactive oxygen species (ROS) (Mehta et al., 1998; Khodr and Khalil, 2001; Pavlick et al., 2002). ROS include oxygen ions, free radicals and peroxides, both inorganic and organic. These molecules are generally very small and are highly reactive, due to the presence of unpaired valence shell electrons. ROS are formed as a natural byproduct of the normal metabolism of oxygen, and play an important role in cell signaling and the induction of host defense genes (Kamata and Hirata, 1999; Dalton et al., 1999). However, under environmental stress, e.g. bacterial infections, ROS levels may increase dramatically, resulting in significant damage to cell structures. This process is known as oxidative stress (Rahman and Macnee, 2000; Wang et al., 2001). Oxidative stress during the viral pathogenesis of insect cell lines has been described previously (Wang et al., 2001). Indeed, oxidative stress is associated with aging and senescence (Arking et al., 2000). In a few studies, some aspects of lipid peroxidation during insect bacteriosis have also been investigated (Boctor and Salama, 1983). Studies showing ROS generation and the antioxidant defence of insects infected by bacteria, however, have been sporadic (Dubovskii et al., 2005).

The cells of native animals are able to defend themselves against ROS damage through the use of antioxidants. Various antioxidants may for example decrease the level of lipid peroxidation as well as DNA and protein damage (Felton and Summers, 1995; Lyakhovich et al., 2006). Major components of the antioxidant defense system of insects include several antioxidant enzymes, such as ascorbate peroxidases (APX), superoxide dismutases (SOD), catalases (CAT), peroxidases (POX), glutathione-*S*-transferase (GST) and nonenzyme antioxidants such as ascorbic acid, thiols, and α-tocopherol (Felton and Summers, 1995). SODs convert superoxide radical $O_2^{\bullet-}$ into $H_2O_2$. CATs and POXs convert the $H_2O_2$ into $H_2O$. In addition, glutathione *S*-transferase (GST) can be considered as an antioxidant enzyme, which can remove the products of lipid peroxidation or hydroperoxides from

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cells (Felton and Summers, 1995; Barbehenn, 2002; Lyakhovich et al., 2006). Thiols defend cells from damage by hydroxyl radical ($OH^{\bullet-}$), nitroxyl radical ($NO^{\bullet-}$), and superoxide radical $O_2^{\bullet-}$ (Udupi and Rice-Evans, 1992). The oxidation of SH-containing compounds results in a decrease in reduced SH-groups (RSH) and an increase in oxidated SH-groups (RSSR). In an organism, increased levels of lipid peroxidation and the thiols ratio (RSSR/RSH) are considered to be markers of oxidative stress (Wang et al., 2001).

The purpose of this study was to test whether Bt infection leads to a change in the balance between ROS production and their inhibition by antioxidants, using the greater wax moth *G. mellonella* as model species. To test this hypothesis we examined the effects of *B. thuringiensis* infection on lipid peroxidation and the GSSG/GSH ratio in the midgut of larvae. In addition, we studied antioxidative responses by determining the activities of SOD, CAT and GST in the larval midgut.

## 2. Methods and materials

### 2.1. Chemicals

The following reagents were used: reduced glutathione, 1-chloro-2,4-dinitrobenzene(DNCB), xanthine oxidase (the activity is 5 U), 5,5-dithiobis-(2-nitrobenzoic acid) (DTNB), 2-thiobarbituric acid (TBA) (ICN, USA), nitroblue tetrazolium (NBT), bovine albumin (BSA) (Sigma–Aldrich, USA).

### 2.2. Insects

Larvae of the greater wax moth *G. mellonella* were reared in glass containers (0.7 L) at 28 °C in the dark, and fed an artificial diet containing bee honey, glycerol, bees' wax and wheat flour (Tamarina, 1987). Sixth instars larvae were used for the experiments.

### 2.3. Insect infection

Larvae of *G. mellonella* were perorally infected by *Bacillus thuringiensis ssp. galleria* strain 69-6 in concentrations of 1.8 $10^9$ spores-crystals/mL, resulting in the mortality of 50% of individuals ($LC_{50}$). This strain, described in an earlier publication (McCord and Fridovich, 1969; Khvoschevskay et al., 2004), has a high virulence against caterpillars of *G. mellonella*. Thirty 6th instar larvae of *G. mellonella* were placed each in their own container with Bt-contaminated diet (3 g of diet mixed with 1 mL of the Bt suspension). An additional fifteen containers were used as a control, in which the food was treated with the same volume of distilled water. After 0 h, 24 h, 48 h and 72 h from the beginning of the experiment, ten to twenty larvae per treatment were randomly selected for each time interval to estimate SOD, Cat, GST activities, MDA and thiol concentrations. Another ten control containers and ten containers treated with Bt were used to test larval weight and mortality. All larvae dead after infection were examined with a microscope to determine the reason of death.

### 2.4. Sample preparation

Midgut dissection was carried out in 10 mM phosphate buffer pH 7.2 containing 150 mM NaCl (PBS). The dissected and washed midgut was homogenized in ice-cold PBS in a proportion of 0.1 g of midgut to 1 mL of PBS. The homogenates were centrifuged for 15 min, 10,000 g at +4 °C. The supernatant was used for spectrophotometric analysis with the Agilent 8453 UV-visible spectroscopy system.

### 2.5. SOD activity

SOD activity was determined as the suppression of the reduction rate of NBT by the superoxide anion, generated as a result of xanthine oxidation by xanthine oxidase (McCord and Fridovich, 1969). Sample (80 μL) was mixed with 500 μL of the reaction solution (70 μM of NBT; 125 μM of xanthine; both dissolved in PBS) and 20 μL xanthine oxidase solution (10 mg of bovine albumin; 100 μL of xanthine oxidase (5.87 units/mL); dissolved in 2 mL of PBS). The mixture was incubated in darkness at 28 °C for 20 min. SOD activity is presented as the difference in absorbance between a sample containing the mixture and a clean reagent mixture at ΔA 560 nm/min/mg protein.

### 2.6. CAT activity

CAT activity was estimated as the decomposition rate of hydrogen peroxide (Wang et al., 2001). Substrate (500 μL 1% hydrogen peroxide in PBS) was mixed with 5 μL of homogenate. The mixture was incubated at 28 °C for 10 min. CAT activity is presented as the ΔA at 240 nm/min/mg protein.

### 2.7. GST activity

GST activity was determined as the changing of the concentration of 5-(2,4-dinitrophenyl)-glutathione (product of DNCB and glutathione interaction) catalyzed by GST (Habig et al., 1974). The reaction mixture contained 1 mM glutathione and 1 mM of DNCB and 20 μL of the sample in 500 μL PBS. The mixture was incubated 5 min at 28 °C. GST activity is represented as ΔA at 340 nm/min/mg protein.

### 2.8. RSSR/RSH ratio

To determine the concentrations of both oxidized (RSSR) and reduced (RSH) thiols, the method based on RSH oxidation by DTNB was used (Khramtsov et al., 1997). Prior to spectrophotometric analysis, the RSSRs were decomposed for 20 min by 1 M hydrochloric acid to form RSH; the pH of the mixture was then neutralized with sodium hydroxide (final pH 7). Fifty microliters of the homogenate was mixed with 500 μL of 0.1% DNTB solution in PBS, and the mixture was incubated for 10 min at 37 °C. The absorbances of RSH and RSH+RSSR were measured at 412 nm. Cysteine was used as a standard to prepare a calibration curve. The concentration of RSSR was calculated as the difference between the final concentration of reduced thiols after reduction by hydrochloric acid (RSH+RSSR) and the initial concentration of one RSH in the sample. The results are presented as the ratio of RSSR to RSH.

### 2.9. MDA concentration

The process of lipid peroxidation results in the formation of MDA. This is a later product in the sequence of lipid peroxidation reactions (Evans et al., 1999; Rael et al., 2004). The TBA assay was used to assess the MDA concentration, with some modifications as described in Bar-Or et al. (2001): 125 μL of 20% trichloroacetic acid was mixed with 250 μL of the sample, after which the mixture was centrifuged at 15,000 g for 10 min at +4 °C. Supernatant (300 μL) was mixed with 200 μL of 0.8 % TBA reagent, and the mixture was incubated at +100 °C for 60 min. The absorbance of the chromophore was measured at 535 nm. The MDA concentration is presented as nmoles of MDA produced per mg protein using a molar extinction coefficient of 1.56 $10^5$ $M^{-1}$ $cm^{-1}$.

### 2.10. Protein concentration

The concentration of protein in the midgut homogenates was determined by the Bradford (1976) method; BSA was used to construct the calibration curve.

### 2.11. Statistical analysis

The data obtained are presented as means±standard error. The Wilk Shapiro W criterion was used to check the normality of sampling. Statistically significant differences were estimated using Student's *t*-test.

## 3. Results and discussion

### 3.1. Bacteriosis of G. mellonella larvae infected with B. thuringiensis

A decrease in the weight of sixth instar wax moth larvae was found when insects were fed on Bt-contaminated diet. During the 3 days of the experiment, larval weight decreased 1.5–2 times ($t=4.90$, $df=18$, $p<0.001$) in comparison with the control insects (Fig. 1). The infected larvae refused feeding already 24 h after the beginning of treatment. The highest mortality of *G. mellonella* larvae from bacteriosis (30±2.1% ($t=-21.12$, $df=18$, $p<0.001$) was registered on the first day after inoculation; a subsequent decrease in mortality to 15±2.5% ($t=7.79$, $df=18$, $p<0.001$) and 0.6±0.6% ($t=11.24$, $df=18$, $p<0.001$) was registered on the second and third days of the experiment respectively (Fig. 1). There was no mortality among the control larvae. It is well known that during bacteriosis the initial acute stage of the disease is the effect of toxins (Broderick et al., 2006). Our results demonstrate that the acute stage of *G. mellonella* bacteriosis occurs 24 h after infection, with maximal larval mortality. The acute period of this bacterial infection could thus serve as a reliable model in the study of oxidative stress in the larval midgut, since it has been well established that oxidative stress in vertebrates is accompanied by acute bacterial-lipid peroxidation-dependent disease (Mehta et al., 1998; Pavlick et al., 2002). The decrease in mortality of the Bt-infected larvae on the second and third day compared to the first day may be due to decreasing toxin concentrations in the gut lumen of the insects because of the refusal of feeding.

### 3.2. Concentration of malondialdehyde

On the first day after the insects were inoculated with Bt suspension, we found a significant ($t=-3.56$, $df=18$, $p<0.001$) increase in the malondialdehyde concentration in the midgut of *G. mellonella* larvae in comparison with the control larvae (Fig. 2). On the second day after the infection, however, MDA concentrations decreased in the larval midgut ($t=4.16$, $df=18$, $p<0.001$) (Fig. 2). On the third day the MDA concentration was the same as that of the control larvae (Fig. 2).

The results obtained testify that the high level of lipid peroxidation on the first day after insect inoculation may be the result of midgut cell destruction by δ-endotoxins. In addition, the formation of lytic pores in the plasmatic membrane and the disconnection of oxidative phosphorylation processes may result in the overproduction of such lipid peroxidation inductors as ROS.

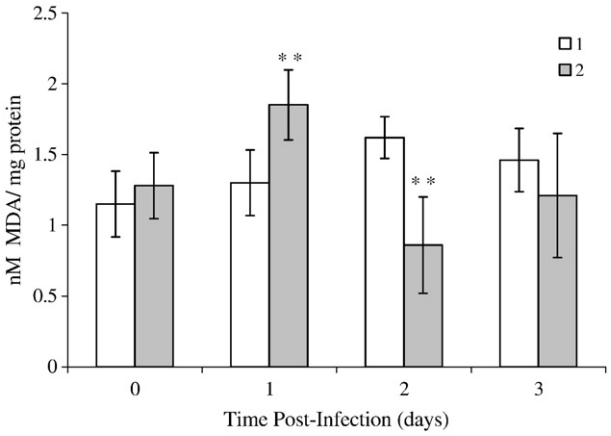


**Fig. 2.** MDA concentration in midgut of native (1) and infected (2) larvae of *G. mellonella*. Asterisks designate statistically significant differences between control and infected larvae (**$p\leq0.001$).

Similar results have been obtained with Bt bacteriosis in *Spodoptera littoralis* (Boctor and Salama, 1983). These studies have reported a reduction in the total lipid number and the quantity of monounsaturated fatty acids in the midgut of infected larvae, while both the quantity of polyunsaturated fatty acids and the concentration of MDA were increased. Lipid peroxidation as a result of oxidative stress during viral infection has also been established in insect cell lines (Wang et al., 2001). The same result was obtained for the effect of plant secondary compounds on insects, where oxidative stress in midgut cells was accompanied by a high level of lipid peroxidation (Peric-Mataruga et al., 1996). We therefore conclude that the increased MDA concentration found in this study during the initial stage of bacterial infection is evidence of oxidative stress. The decrease in lipid peroxidation on the second day may be due to a decrease in toxin content in the gut during the insect's starvation, as well as to an increase in antioxidant defense.

### 3.3. Concentration of oxidized and reduced thiols

We have noted the significant rise ($t=-2.41$, $df=18$, $p=0.027$) of the RSSR/RSH ratio in the midgut of infected insects on the first day of the experiment in comparison with the controls (Fig. 3). The change of balance to oxidized thiol production (the increase in the RSSR/RSH ratio) during bacteriosis may be evidence of increased activity of

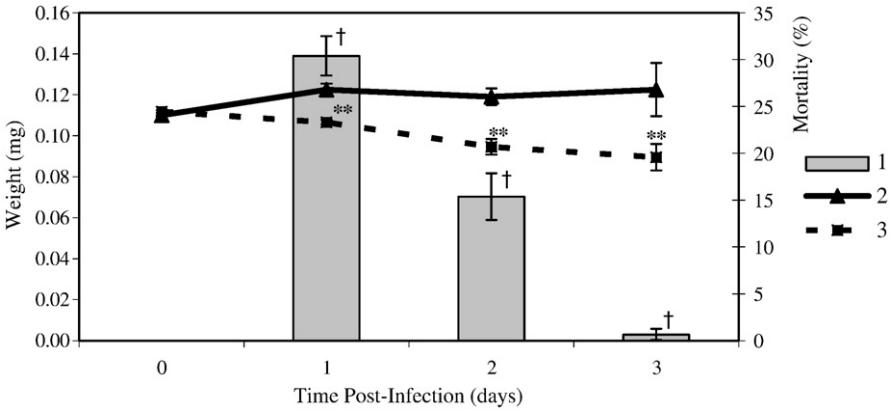


**Fig. 1.** Mortality (1) and weight of infected (2) and control (3) larvae of *G.mellonella*. In the control treatment mortality was absent. Asterisks designate statistically significant differences between values for weight on the same day (**$p\leq0.001$). Daggers designate statistically significant differences in mortality on different days after infection (†$p\leq0.001$).

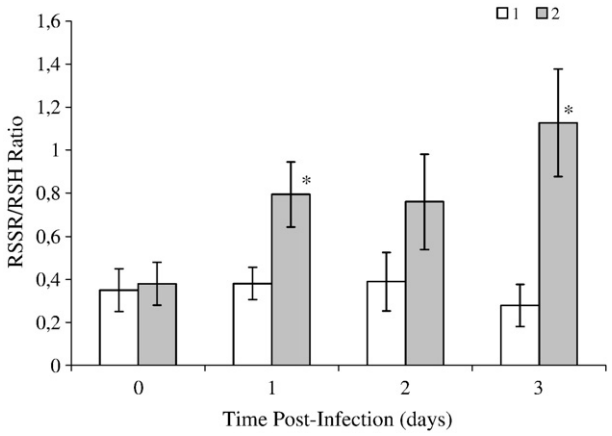


**Fig. 3.** Ratio of concentrations of oxidized to reduced thiols (RSSR/RSH) in midgut of native (1) and infected (2) larvae of *G. mellonella*. Asterisks designate statistically significant differences between control and infected larvae (*$p<0.05$).

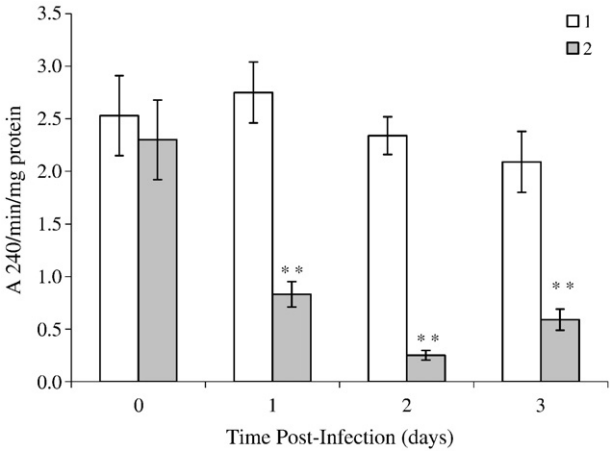


**Fig. 5.** Catalase activity in midgut of native (1) and infected (2) larvae of *G. mellonella*. Asterisks designate statistically significant differences between control and infected larvae (**$p \leq 0.001$).

radical oxidative processes. An increase in the ratio of oxidized to reduced thiols has been demonstrated, together with an increase in lipid peroxidation processes against a background of an increase in cell death (Wang et al., 2001). It is possible that the increased RSSR/RSH rate during the initial stage of bacterial infection may be further evidence of oxidative stress.

### *3.4. Activity of glutathione-S-transferase, superoxide dismutase and catalase*

We found increased SOD activity in the midgut of Bt infected larvae on the first ($t=-4.76$, $df=33$, $p<0.001$), second ($t=-2.81$, $df=24$, $p=0.0094$) and third ($t=-2.28$, $df=29$, $p=0.03$) day of the experiment (Fig. 4), while CAT activity decreased at the same time: first day $t=6.04$, $df=38$, $p<0.001$; second day $t=10.28$, $df=36$, $p<0.001$; third day $t=4.84$, $df=37$, $p<0.001$ (Fig. 5). GST activity in the midgut of infected larvae was significantly increased on the first day ($t=-2.12$, $df=34$, $p=0.041$) and the third day ($t=-4.21$, $df=34$, $p<0.001$) after inoculation in comparison with the control (Fig. 6).

These results demonstrate that enhanced activities of SOD and GST can lead to the elimination of ROS. The increase in GST activity in the larval midgut on the first day of the experiment was observed simultaneously with the increased concentration of malondialdehyde. This finding may prove that GST is involved in the inactivation of toxic lipid peroxidation products accumulated during destructive processes in the insect midgut in the early stage of bacteriosis. In particular, as other authors have shown, GST may eliminate organic hydroperoxide from cells and defend cells from potential damage from the products of lipid peroxidation (Morrissey and O'Brien, 1980).

It should be noted that SOD and CAT together take part in stepwise oxygen reduction (Munday and Winterboume, 1989; Sies, 1991). Since SOD activity was enhanced in the infected larvae, we assumed that this increased SOD activity would result in an increased $H_2O_2$ concentration and consequently in a further increase in CAT activity. However, catalase activity in infected *G. mellonella* larvae was significantly decreased during the whole experimental period (Fig. 5). CAT is known to be inhibited by the accumulation of superoxide anion during destruction processes in the gut (Kono and Fridovich, 1982; Pardini et al., 1988). Another study concerning SOD and catalase activities in the midgut of *Lymantria dispar* larvae fed on an unfavorable plant has shown similar results (Peric-Mataruga et al., 1996). The authors registered a decrease in catalase activity together with an increase in SOD activity. We therefore assume that CAT activity was inhibited as a result of the high level of superoxide radical generation during oxidative stress in the acute stage of bacteriosis.

In conclusion, we assume that oxidative stress is a syndrome of bacterial disease and is involved in intensifying a radical related

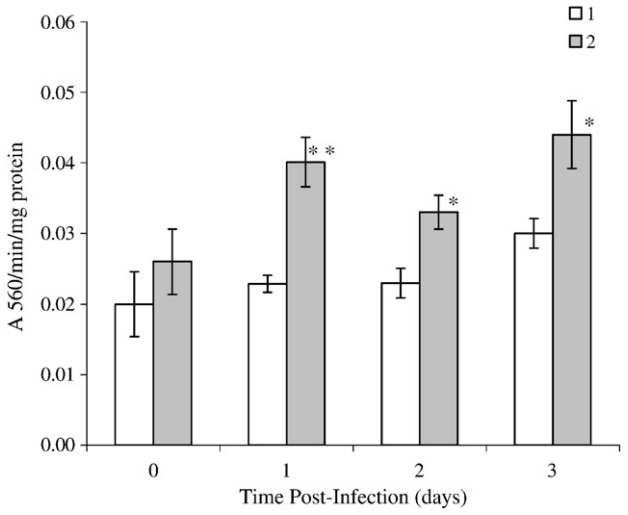


**Fig. 4.** SOD activity in midgut of native (1) and infected (2) larvae of *G. mellonella*. Asterisks designate statistically significant differences between control and infected larvae (*$p \leq 0.05$; **$p \leq 0.001$).

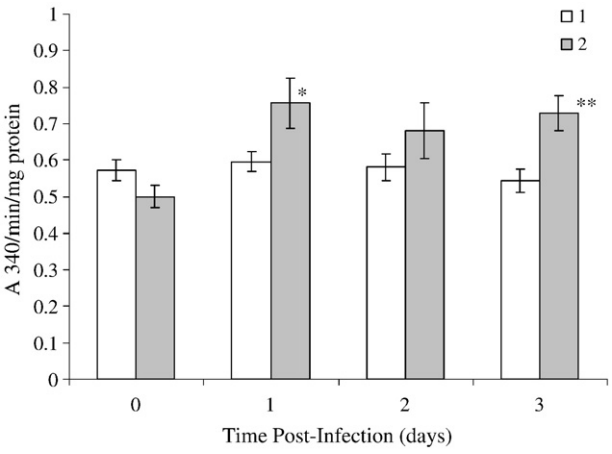


**Fig. 6.** GST activity in midgut of native (1) and infected (2) larvae of *G. mellonella*. Asterisks designate statistically significant differences between control and infected larvae (*$p \leq 0.05$; **$p \leq 0.001$).

process, in particular lipid peroxidation. We have confirmed that Bt toxins may disturb the functional activity of cell membranes and intensify the activity of lipid peroxidation processes. The effect of oxidative stress is probably a nonspecific cytotoxic instrument of Bt action, which in the acute stage of bacteriosis forms lytic pores and destroys the structure of the insect gut. Consequently, increased oxidative stress leads to an up-regulation of antioxidants, such as the RSSR/RSH rate, SOD and GST. On the other hand, these processes may be mirrored in insect physiological adaptations and resistance to Bt, particularly protein modification, changes in the synthesis of specific larval gut proteins, and increased oxidative metabolism (Loseva et al., 2001; Ferre and Van Rie, 2002; Candas et al., 2003). Oxidative stress during bacteriosis is probably a metabolic adaptation by insects, and may mediate detoxification as well as higher rates of generalized and localized mutations, which in turn enhance the insect's resistance to Bt and provide a survival advantage.

When the consumption of toxins was decreased after defensive starvation of the insects, we found a decrease in lipid peroxidation against a background of increased antioxidant activity and decreased larvae mortality. We therefore assume that the activity of the antioxidant system is one of the defense mechanisms involved in the repair and stabilization of the oxidation-reduction balance in the insect midgut during bacterial pathogenesis.

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