Changes in vitellogenin expression caused by nematodal and fungal infections in insects

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Summary statement

This study revealed changes in mRNA and protein expression levels of insect vitellogenin caused by nematodal and fungal infections. Vitellogenin inhibited growth of entomotoxic bacteria from the nematode *Steinernema carpocapsae*.

Abstract

This study examined the expression and role of vitellogenin (Vg) in the body of the firebug *Pyrrhocoris apterus* (Heteroptera, Insecta) during the infection elicited by two entomopathogenic organisms, the nematode *Steinernema carpocapsae* and the fungus *Isaria fumosorosea*. Infection by *S. carpocapsae* significantly up-regulated *Vg* mRNA expression in the male body. The corresponding increase in Vg protein expression was also confirmed by

electrophoretic and immunoblotting analyses. Remarkably, in females, the tendency was opposite. Nematodal infection significantly reduced both *Vg* mRNA and Vg protein expression levels in fat body and hemolymph, respectively. We speculate that infection of reproductive females reduces Vg expression to the level, which is still sufficient for defense, but insufficient for reproduction. This circumstance reduces energy expenditure and helps the individual to cope with the infection. Importantly, purified Vg significantly inhibited growth of *Xenorhabdus* spp., an entomotoxic bacteria isolated from *S. carpocapsae*. However, the effect of Vg against *I. fumosorosea* was not so obvious. The fungus significantly stimulated *Vg* gene expression in males, however, a similar increase was not recapitulated on the protein level. Nevertheless, in females, both mRNA and protein Vg levels were significantly reduced after the fungal infection. The obtained data demonstrate that Vg is likely an important defense protein, possibly with a specific activity. This considerably expands the known spectrum of Vg functions, as its primary role was thought to be limited to regulating egg development in the female body.

Key words: insect, vitellogenin, infection, entomopathogenic nematode, entomopathogenic fungus, antimicrobial activity

INTRODUCTION

Vitellogenins (Vgs), glycolipophosphoproteins mostly known and well-characterized as precursors of yolk proteins, are involved in the reproduction in the majority of oviparous animals. In insects, Vgs are typically synthesized in the fat body, from where they are transported via hemolymph into growing oocytes. After they enter the oocyte by endocytosis via specific receptors, Vgs usually undergo some modifications, transforming into vitellins. Most insects produce only one or two types of Vgs that comprise several subunits with the total molecular weight ranging from 150 to 650 kDa. Vg production is hormonally controlled. It is known for decades that juvenile hormone stimulates Vg synthesis in most insect species (see Chapman, 1998). Furthermore, the termination of Vg synthesis is controlled by adipokinetic hormone that inhibits the synthesis of the protein part of the Vg molecule directly in the fat body (Carlisle and Loughton, 1986). Besides this, Vg production is controlled by the nutrient levels and mating status (Chapman, 1998).

Vgs are typically present in egg-laying females. However, low levels of Vgs have been identified in males of several insect species, including the firebug *Pyrrhocoris apterus* (Němec et al., 1993). Several recent studies suggested that Vgs play an important role not

only in the reproduction, but also in other aspects of insect biology, such as caste differentiation process in social insects, wound healing, protection against oxidative stress, immunity, and life span regulation (Havukainen et al., 2013; Singh et al., 2013; Salmela et al., 2015; Salmela and Sundstrom, 2017; Park et al., 2018). Studies in the silkworm and honeybees reported strong antibacterial activity of Vg against gram-positive and gram-negative bacteria (Singh et al., 2013), showing that Vg bound to bacterial cells and destroyed them. Vg was active even against *Paenibacillus larvae,* a gram-positive bacterium infesting young honeybee larvae and causing a disease called the American foulbrood, probably the deadliest bee brood disease worldwide (Salmela et al., 2015). In addition, it has been reported that infection of honey bee larvae by the spores of the microsporidium *Nosema ceranae* significantly up-regulated Vg expression in workers (BenVau and Nieh, 2017; Sinpoo et al., 2018), and that bee Vg interacted with the cell wall of the entomopathogenic fungus *Beauveria bassiana,* eliciting membrane disruption and permeabilization. Furthermore, Vg appears to induce trans-generational immune priming in bee queens, enhancing immunity in their offspring by transporting pathogen-associated pattern molecules, which are attached to Vg, into the eggs within queen ovaries (Sadd et al., 2005; Salmela et al., 2015).

Oxidative stress is caused by the accumulation of reactive oxygen species primarily produced within mitochondria as unavoidable aerobic metabolism by-products (Beckman and Ames, 1998). The anti-oxidative response has evolved a suite of defense mechanisms, involving both enzymatic and non-enzymatic components (Fridovich, 1978) controlled by adipokinetic hormones in insects (Krishnan et al., 2007; Bednářová et al. 2013; Kodrík et al., 2015). Vg plays an important role in this process as it has been shown to elicit anti-oxidative protection against oxidative stressors such as paraquat or hydrogen peroxide (Seehuus et al., 2006; Park et al., 2018). It was proposed that anti-oxidative effect of Vg might be a crucial mechanism that extends the life span of honey bee long-lived winter workers and queens, in which Vg is synthesized in high levels. However, exact mechanisms of the anti-oxidative effects of Vg and hormones in insect body remain unclear.

In the present study, we sought to examine Vg role in the defense against two different entomopathogens: the nematode *Steinernema carpocapsae* and the fungus *Isaria fumosorosea*. The nematode *S. carpocapsae* carries symbiotic *Xenorhabdus* spp. bacteria that are toxic for insects (Simões et al., 2000; Duchaud et al., 2003), and the nematobacterial complex represents an efficient tool for insect killing commonly used for insect pest control (Ehlers, 2003; Inman et al., 2012). Similarly, the fungus *I. fumosorosea,* harbored by the horse chestnut leaf miner *Cameraria ohridella,* plays a significant role in the biological

control of many insect species (Zimmermann, 2008).The main aim of the present study was (1) to examine changes in Vg gene and protein expression upon the infection with entomopathogenic nematode (EPN) and entomopathogenic fungus (EPF); (2) to elucidate the role of Vg in insect body infected by EPN, and (3) to determine whether Vg-mediated defense reaction in response to EPF, similar to that observed in honey bees or silkworms (see above), is a common defense mechanism in insects. In addition (4), we sought to explain the role of Vg in insect males.

MATERIAL AND METHODS

Experimental insects

A stock culture of the firebug *P. apterus* (L.) (Heteroptera), established from wild populations collected at České Budějovice (Czech Republic, 49 ◦N), was used for the present study. Larvae and adults of the reproductive (brachypterous) morph were kept in 500 ml glass jars in a mass culture and reared at constant temperature of 26 ± 1°C under long-day conditions (18 : 6 h light : dark). They were supplied with linden seeds and water *ad libitum*, which were replenished twice weekly. Female and male adults were kept separately (Socha and Kodrík, 1999).

Entomopathogenic nematode *Steinernema carpocapsae* and the insect treatment

The nematodes *S. carpocapsae* originating from Russia (strain NCR), St. Petersburg were obtained by courtesy of Dr. Z. Mráček (Institute of Entomology, České Budějovice). They were reared under laboratory conditions using the last larval instar of *Galleria mellonella* (Lepidoptera, Insecta) as a host. The emerging infective juveniles were harvested and subsequently stored in water at 4°C for 30 days. Their viability was confirmed under a microscope before experiments.

For vitellogenin (Vg) experiments 7-day old males and 1 - 4-day old females were treated individually with *S. carpocapsae* by injection into the hemocoel with 10 nematodes in 2 µl autoclaved water per individual; controls were injected by autoclaved water only. The firebugs were transferred into the glass jars and kept under the same condition as for the stock culture. Hemolymph of surviving individuals was collected in 1 day after their infection and stored at -20°C until used.

Entomopathogenic fungus *Isaria fumosorosea* and the insect treatment

The fungus *I. fumosorosea* isolate originating from the horse chestnut leaf miner, *Cameraria ohridella*, Decka and Dimic (Lepidoptera, Gracillariidae) was obtained by courtesy of Dr. A. Bohatá (Agricultural Faculty, South Bohemian University, České Budějovice). The strain is deposited under the number CCM 8367 as a patent culture in the Czech Collection of Microorganisms in Brno (Prenerová et al., 2009). The spore suspensions was prepared by scraping of 14-day old conidiospores into the sterile solution of 0.05% (v/v) Tween®80 (Sigma-Aldrich). Suspension was filtered through sterile gauze to separate the mycelium and clusters of spores. In uniform suspension, the number of spores was counted with a Neubauer improved chamber and subsequently the suspension was adjusted to concentration 1×10^7spores per ml. Conidial suspension (5 ml) was added to 100 ml potato dextrose broth in 250 ml Erlenmeyer flask. After inoculation, Erlenmeyer flask was placed on a shaker and incubated at 25°C under a shaking (200 rpm) and constant light. In 4 days the blastospores were harvested and injected into 7-day old males and 1-day old females in a dose of 30,000 blastospores/bug; controls were injected by the Ringer saline only. The firebugs were transferred into the glass jars and kept under the same condition as for the stock culture. Hemolymph of the surviving individuals was collected in 1-3days after their infection and stored at -20°C until used.

RNA and cDNA preparation, primers

- *The fat body preparation*. *P. apterus* males treated by injection of *S. carpocapsae* or *I. fumosorosea* were collected and stored at -80°C prior processing. To monitor expression profile of the *Vg* gene, the fat body was dissected under a stereomicroscope on sterilized glass Petri dishes placed on crushed ice and in sterile, ice-cold RNAase-free Ringer solution. Fat bodies of four *P. apterus* individuals were pooled as one replicate, and four biological replicates per tissue of control and nematode treated *P. apterus* males were generated. Immediately after dissection the fat bodies were transferred to microcentrifuge tubes with 200 µl of TRI Reagent® (Sigma-Aldrich) on crushed ice and then stored at −80 °C until RNA isolation.

- *RNA isolation and cDNA synthesis.* The total RNA was extracted using TRI Reagent® (Sigma-Aldrich) following the manufacturer's protocol. RNA isolates were treated with RQ1 RNase-Free DNase (Promega) to remove traces of contaminant DNA. The cDNA template was prepared using the SuperScript® III First-Strand Synthesis System for RT-PCR

(Invitrogen by Life Technologies) on 2 µg of the corresponding total RNA with random hexamers.

Quantification of *Vg* gene expression

Quantitative real-time PCR (qReal-time PCR) was performed to evaluate *Vg* transcript levels in the fat bodies of the experimental firebugs. For those studies the same experimental designs (age of male and female firebugs, schedule of infection by the nematode and fungus, time table etc.) as for analysis of Vg protein in hemolymph were used (see above). The experiments were accomplished on a Light Cycler CFX96 BioRad real-time PCR system using Xceed qPCR SG 2x Mix Lo-ROX (Institute of Applied Biotechnologies), and relative levels of *Vg* transcripts were determined using the threshold cycle and normalized to *Rp49* (*Ribosomal protein 49*). Primers used for qReal-time PCR were: Vg (forward) CCCGACAAGTCCACAGTTATT, Vg (reverse) GCGCATTCTGTTCATGTAAGC, Rp49 (forward) CCGATATGTAAAACTGAGAAAC, and Rp49 (reverse) GGAGCATGTGCCTGGTCTTTT.

Gel electrophoresis and vitellogenin quantification

Electrophoresis under denaturing conditions using sodium dodecylsulphate polyacrylamide (SDS-PAGE) commercial gels (Bio-Rad, 5-20%) was performed according to Laemmli (1970) as modified by Socha et al. (1991). Typically, hemolymph samples were diluted in a sample buffer by 10-fold and 25-fold in the male samples and female samples, respectively, and 10 µl each used for the analysis. The proteins separated on gels were stained with Coomassie Brilliant Blue R-250, and the Vg bands determined according to the molecular weight (MW) standards (10 - 250 kDa, Thermo Fishers Scientific) and according to a reaction with a specific antibody (see below); their quantities were evaluated on the GS-800 Calibrated Densitometer using Quantity One (Version 4.6) software (Bio-Rad).

Western blotting

After SDS-PAGE the separated proteins were blotted onto nitrocellulose membrane according to Towbin et al. (1979). Specific antibody against *P. apterus* Vg (1: 1000 v/v; Socha et al., 1991) was used in the procedure including the secondary antibody Goat/HRP (1:1000 v/v; goat anti rabbit labelled with horse radish peroxidase; Sigma-Aldrich). For visualization the Novex® ECL HRP chemiluminescent substrate reagent kit (Invitrogen) in A and B solutions

1:1 v/v was used. The developed color was documented using Intelligent Dark Box (LAS 3000, Fujifilm).

Vg isolation and its antimicrobial activity

The crude Vg was isolated from hemolymph of 3 - 4-day old *P. apterus* females to test its antimicrobiological activity. The hemolymph samples were separated by polyacrylamide electrophoresis using 10 % gel according to Laemmli (1970) similarly as mentioned above. After that the gel was stained with a low concentration of Coomassie Brilliant Blue R-250 (0.05 %) as recommended by Harlow and Lane (1988). After de-staining, visualized Vg bands were excised from the gel by scissors (1.5 mm gel, 10 wells) and electroeluted overnight using Electro-Eluter (Bio-Rad) in a volatile ammonium bicarbonate buffer. Simultaneously, a gel strip containing no Vg was processed as control. The samples were evaporated to dryness, solved in a Ringer saline, and their protein content quantified by the Bicinchoninic Acid Protein Assay Kit (Sigma-Aldrich) (Stoscheck 1990). The bovine serum albumin standard curve was used to convert the optical densities of the samples measured at 562 nm to μg proteins. Thereafter the samples were stored at -20^{o} C until needed.

For the Vg antimicrobial tests the disc diffusion method using *Xenorhabdus* spp bacterium was employed; this entomotoxic organism is symbiotically associated with the nematode *S. carpocapsae*. The bacteria were isolated from the larvae of the greater wax moth, *Galleria mellonella* infected with infective juveniles of *S. carpocapsae* according to Mahar et al. (2005). The dead *G. mellonella* larvae were surface-sterilized in 75% alcohol for 10 min and opened with sterile needles and scissors. Then, a drop of the leaking hemolymph was streaked with a needle onto MacConkey Agar plates. The plates were incubated at 30 °C in the dark for 24 h, and then a single bacterial colony was selected and streaked onto new plate of MacConkey Agar and finally used for inoculation of 2% LB broth (Lennox) solution. The inoculated solution was shaken at 150 rpm for 1 day at 30 °C. Next day the density of bacterial suspension was adjusted to be 0.8 McF (McFarland bacterial density), and 0.2 ml were swabbed onto the agar plates. Vg (about 40 μg) was applied onto a sterile paper discs (Sigma-Aldrich) dried in a laminar airflow cabinet, and placed on the bacterial lawns. By the same way also the gel extract (without Vg - see above) and diluting buffer (Ringer saline) were applied as controls. The plates were incubated at 30 °C overnight, zones of growth inhibition around the paper discs were measured and their area calculated.

Mortality test

Mortality test with *S. carpocapsae*, using an assay described previously by Ibrahim et al. (2017) with some modifications, was employed to evaluate possible differences between the firebug males and females. Briefly, we used 7-day old males and 4-day old females, and each of the tested individual was infected by injection of 10 nematodes in 2 µl autoclaved water into the hemocoel; controls were injected by the Ringer saline only. To determine mortality, five groups (each consisting of 20 firebugs) for each experimental treatments as well as for controls, were inspected 24 hours post treatment.

Similarly, the effect of *I. fumosorosea* on mortality of the firebug males and females was examined. The firebugs were injected by a dose of 30,000 blastospores/bug; controls were injected by the Ringer saline only. The mortality was monitored in 1-3-day post infection.

Data presentation and statistical analysis

The results were plotted using the graphic software Prism (Graph Pad Software, version 6.0, San Diego, CA, USA). The bar graphs represent mean ± s.d., the numbers of replicates (n) are depicted in the figure legends. The statistical differences were evaluated by Student's t-test (Figs. 1, 2, 5, 6, 8 and 10), and two-way ANOVA (Figs. 3, 4, 7, 9 and S2) and one-way ANOVA (Fig. S1) using the Prism software.

RESULTS

***Steinernema carpocapsae* infection**

The first series of experiments focused on measuring *Vg* transcript levels during nematobacterial infection of the fat body of male *P. apterus*. Infection of males with the EPN *S. carpocapsae* resulted in a 7.7-fold increase of *Vg* transcript level on the next day post infection (Fig. 1). Vg protein level in hemolymph was also increased 1.5-fold, as visualized by PAGE (Fig. 2A, C). These observations were then verified using western blot with an anti-Vg antibody (Fig. 2B). Interestingly, the anti-Vg antibody used in western blot positively recognized not only the main Vg band (about 180 kDa), but also two smaller bands of 65 and 24 kDa, which might be degradation products of EPN toxic actions in the body of *P. apterus* males.

As expected, *Vg* gene expression level was substantially higher in the fat bodies of females than in those of males (Fig. 3). *Vg* transcript levels in females continuously increased during the first 5 days of development (Fig. 3), i.e., the critical period for egg formation.

Application of EPN radically reduced the level of Vg transcripts in 2–5-day old females on the next day (Fig. 3); the extent of inhibition ranged from about 9- (in 4-day-old) to about 150-fold (in 3-day-old females). A similar trend was observed with Vg protein levels in hemolymph, although the differences were not so profound (Fig. 4): 12-fold change (in 3-day old females) was recorded as the maximal one (Fig. 4C). These results were confirmed by immunoanalysis using western blotting (Fig. 4B). In contrast to the reaction observed in males, the antibody recognized only the 180 kDa Vg band in female hemolymph. This suggests that male and female bodies reacted differently to EPN infection.

S. carpocapsae elicited mortality in the treated firebugs (Fig. 5A). The mortality rate was about 2.9 times lower in females than in males in 1 day after the treatment. In corresponding controls, no mortality was recorded (data not shown), maybe due to a higher level of Vg in the female body. Further, using the disc diffusion method, we tested the antimicrobial effect of Vg on growth of the bacterium *Xenorhabdus* spp. isolated from *S. carpocapsae* body (Fig. 5B). We found that Vg inhibited *Xenorhabdus* growth; the inhibition was 2.5-fold more effective than in controls.

***Isaria fumosorosea* infection**

Due to slower development of the EPF *I. fumosorosea* in the fire bug body, Vg levels were monitored daily for 2-3 days after the infection in the tested individuals. In the male fat body, *Vg* transcript level nominally increased 1.6-fold on the next day after infection, although this change did not reach the level of statistical significance (Fig. 6). However, on day 2 after the infection, the 2-fold increase in *Vg* transcript level was significant. Nevertheless, the infection had no impact on Vg level in hemolymph (Fig. 7), as PAGE and western blotting results were not significantly different between EPF infection and control groups.

Females exhibited a more pronounced response to EPF infection. Strong reductions of *Vg* transcript level in the fat body was recorded on both the first and second days after the infection (Fig. 8). Similar significant changes in Vg protein level were detected in hemolymph during the whole monitored period according to PAGE analysis (Fig. 9A, C). Identical results were obtained also using immunoblotting (Fig. 9B). Surprisingly, no differences between male and female firebugs were recorded when mortality was monitored for 1–3 days after the treatment with *I. fumosorosea* (Fig. 10).

DISCUSSION

Pathogenic organisms elicit severe stress in the host body, which results in the disruption of functional homeostasis (Ivanovič and Jankovič-Hladni, 1991) and activation of characteristic defense response to eliminate or at least to reduce the impact of the stress on the organism. This response occurs at both organismal and cellular levels (Hightower, 1991), and may include both humoral and cellular defenses. The humoral response includes production of various compounds, such as eicosanoids, phenoloxidases, proteinases, proteinase inhibitors, and a wide selection of antimicrobial peptides and proteins (Jiang, 2008; Beckage, 2008). The list of the protective compounds includes also Vgs, whose involvement into insect defense system has recently been described in several insect species (Havukainen et al., 2013; Singh et al., 2013; Salmela et al., 2015; Salmela and Sundstrom, 2017; Park et al., 2018). We have found in this study that in the firebug *P. apterus*, Vg is probably also involved into the defense reaction against the entomopathogenic nematode *S. carpocapsae* and, partially, against the fungus *I. fumosorosea*.

To invade their hosts, EPNs usually use oral and anal openings, or spiracles. To speed the infection up, we injected *S. carpocapsae* into hemocoel. Once the nematodes are inside the insect body, the infection develops quite quickly, therefore we monitored the effect of the EPN just in 1 day post infection: on the next day after that, the mortality reached almost 100% (data not shown). During the development in the host body, EPNs produce various venoms and toxins, which are generated by nematodes themselves and also by symbiotic bacteria (Simões et al., 2000; Duchaud et al., 2003). In the first step of the nematobacterial infection, the toxins protect EPNs against the defense system of their insect host, and afterwards, they kill the host, whose organs are then utilized as a source of nutrients for EPN growth and development. The insects protect themselves by clotting cascades, production of reactive oxygen species, and other fast-reacting immune factors (Wang et al., 2010; Hyršl et al., 2011; Arefin et al., 2014; Kodrík et al., 2015).

We have found in this study that the nematobacterial complex of *S. carpocapsae* and *Xenorhabdus* spp. affected Vg characteristics in both male and female *P. apterus*. In males, a significant stimulatory effect of the infection was noted on both *Vg* transcript level in the fat body and on Vg protein level in hemolymph. To the best of our knowledge, this is the first report about a stimulatory effect of the nematobacterial complex on Vg production in infected insects. However, at this point, we cannot state, based on our results, whether the effect was primarily elicited by the nematode, its symbiotic bacteria *Xenorhabdus* spp., or by the

combined effect of the exposure to both of them. However, it is known that both organisms are insect pathogens (Herbert and Goodrich-Blair, 2007; Waterfield et al., 2009). Further, it looks that interaction of male Vg with EPN results not only in the stimulation of Vg synthesis, but also in the degradation of Vg molecules. Immunoblotting results clearly showed at least two products that positively reacted with the anti-Vg antibody with molecular weights (24 and 65 kDa) well below the molecular mass of 180 kDa of the intact protein. All these results suggest an active role of Vg against EPN infection in the male body. It is obvious that Vg plays a key role as an irreplaceable component of yolk in eggs developing in the female body, however Vg role in immunity, perhaps secondary, seems to be important as well. Evidence about the presence of Vg in male body has been rather scarce, but Vgs have been identified in *P. apterus* (Němec et al., 1993), *Apis mellifera* (Vilar and Grozinger, 2017), and *Bombus terrestris* (Jedlička et al., 2016) males. Nevertheless, a comprehensive understanding of Vg role in insect males is missing.

Furthermore, the multi-faceted role of Vg in insect female body is apparently more complicated: EPN infection significantly decreased both *Vg* mRNA and Vg protein levels. Although it is surprising that EPN infection caused opposite effects in male and female *P. apterus*, on the other hand, this fact does not need to be so incomprehensible: one can speculate that Vg level sufficient for effective defense against pathogens might be much lower than that required for nutritional supply of developing eggs. Thus, during the infection, the female body simply shuts down less important processes to save energy for more significant activities. This trade-off strategy is not so exceptional in insects facing various stress situations. For example, the resistance of females of the corn earworm *Helicoverpa armigera* against *Bacillus thuringiensis* toxin Cry1Ac was accompanied by the inhibition of reproduction caused by a decrease in *Vg* gene expression (Zhang et al., 2014; 2015). Similarly, in females of the rice stem borer *Chilo suppessalis*, application of sublethal doses of the insecticide chlorantraniliprole reduced Vg mRNA expression (Huang et al., 2016). Additionally, adipokinetic hormone, responsible for energy mobilization at the time of its increased consumption, suppresses less important processes when the organism is under stress and, in certain conditions, even draws on the mobilized energy (Kodrík, 2008). Moreover, in *Locusta migratoria*, adipokinetic hormone inhibits Vg production in the end of female reproductive cycle (Moshitzky and Applebaum, 1990). This process is independent of nutrient mobilization because Vg inhibition occurs at hormone titers about one tenth of those necessary for nutrient mobilization from the fat body: thus, the two activities stimulated by adipokinetic hormone are not overlapping (Carlisle and Loughton, 1986). Anyway, the

mechanism of Vg functions during infection is unclear, and perhaps different in males and females – for example, no Vg degradation products were observed by immunoblotting in female hemolymph during infection.

We have demonstrated a bactericidal effect of Vg on the bacterium *Xenorhabdus* spp. isolated from *S. carpocapsae*. This clearly suggests that Vg has a certain protective role against the nematobacterial complex, because Vg likely kills entomotoxic bacteria. It has not been established whether Vg affects EPNs, therefore, we cannot definitely exclude this. An antibacterial effect of Vg had already been described in several studies. Singh et al. (2013) showed that Vg of the silkworm *Bombyx mori* had antibacterial activity against the gram-positive bacterium *Bacillus subtilis* and the gram-negative bacterium *Escherichia coli*. Furthermore, Vg of *Apis cerana* was active against *E. coli,* and also against the gram-positive bacterium *B. thuringiensis* (Park et al., 2018). In the latter example, Vg bound to bacterial surface, inducing structural damage in the cell wall, which resulted in membrane disruption and permeabilization. Thus, all those data suggest that Vg is an antibacterial agent with wide spectrum of action.

EPFs, such as *I. fumosorosea* used in this study, usually start their infection by breaking the host cuticle and physically penetrating into the host body. For that, they use various enzymes, such lipases, proteases, chitosanases, and chitinases (Hajek and Leger, 1994; Ali et al., 2010). In the host body, EPFs use these enzymes to dissolve tissues and organs, and the resulting matter is then utilized as a source of nutrition for EPF growth. In *I. fumosorosea*, the process is facilitated by the production of beauvericin, a toxic depsipeptide that kills the infected cell (Luangsa-Ard et al., 2009). Despite those effective mechanisms, the whole process of EPF infection is rather slow — to speed it up, we used the injection of blastospores, which was a similar approach to the injection of EPNs (see above). Nevertheless, the EPF infection developed more slowly than the EPN one. However, this circumstance enabled monitoring EPF effect for 2-3 days after the injection. The response of *P. apterus* male body to EPF infection differed from that to the infection with EPN. The first significant up-regulation of *Vg* transcription in the fat body was observed in 2 days post infection, however, surprisingly, *Vg* mRNA levels in hemolymph did not show the same trend, as similar *Vg* mRNA expression was recorded in infected and control males. Additionally, the pattern of Vg protein level changes in male hemolymph, as determined by immunoblotting, was apparently different from the results obtained after EPN infection (compare Figs. 2B and 7B). This suggests different responses of the male body to these infections: the involvement of Vg in the defense reaction is apparently less intensive in the case of EPF infection. It remains to be

determined whether any other defense systems are involved into responses to infections with EPN and EPF. In contrast, the response of firebug females to the EPF infection was quite similar to that elicited by EPN: both mRNA and protein Vg levels were significantly down-regulated. Similar reaction was recorded in the whitefly *Bemisia tabaci*, in which both *I. fumosorosea* mycelium (*in vivo*) and fungal extracts (*in vitro*) showed a decrease in Vg level, and corresponding damage of the ovaries (Gao et al., 2017). Possible reasons for this phenomenon have already been discussed in the previous paragraph. In addition, active involvement of Vg into the defense reaction of *A. mellifera* against the entomopathogenic fungus *B. basiana* was recently described by Park et al. (2018), who demonstrated that Vg behaves as a typical anti-microbial peptide. *A. mellifera* Vg bound to *B. basiana* cells and induced structural damage of the cell wall leading to anti-microbial activity against the fungus. Interestingly, Vg is also present in the venom of the honey bee and some other hymenopterans (Blank et al., 2013; Park et al., 2018), where it probably serves as an allergen that intensifies venom efficacy by causing allergic reaction in the stung tissue. Vg is also expressed in the brain of several hymenopteran species, where it controls various processes, including aging (Munch et al., 2015; Lockett et al., 2016; Gospocic et al., 2017).

In conclusion, our findings expand the growing list of Vg functions in insects, which are more complex than previously thought. It is obvious that Vg proteins play an important role in the defense against various types of infections, including those caused by EPN and EPF. Furthermore, Vg transcription and protein synthesis are modulated depending on the type of the infection and the sex of infected insect. Vg acts directly against *Xenorhabdus spp*. bacteria isolated from the entomopathogenic complex of *S. carpocapsae*. Other mechanisms of Vg described activities are not known at present, but they might differ depending on the type of infection, as documented by different Vg characteristics (in males) and different mortality response (in both sexes) to the two studied pathogens.

Acknowledgements

The authors thank Mrs. H. Štěrbová and Miss D. Hlávková for their technical assistance. The English grammar and stylistics were checked by the Editage Author Services.

Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: D.K.; Methodology: E.I., U.K.G., V.K., P.J.; Validation: D.K., R.Č.F.; Formal analysis: D.K., A.B., R.Č.F.; Investigation: D.K., R.Č.F., A.B., P.J.; Writing – original draft: D.K.; Writing – review & editing: D.K., RČF; Supervision: D.K.; Project administration: D.K.; Funding acquisition: D.K.

Funding

This study was supported by grant No. 17-03253S (DK) from the Czech Science Foundation, and by projects RVO 60077344 of the Institute of Entomology. AB acknowledges Fellowship No. L200961701 from the Program of Support of Promising Human Resources, awarded by The Czech Academy of Sciences.

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Figures

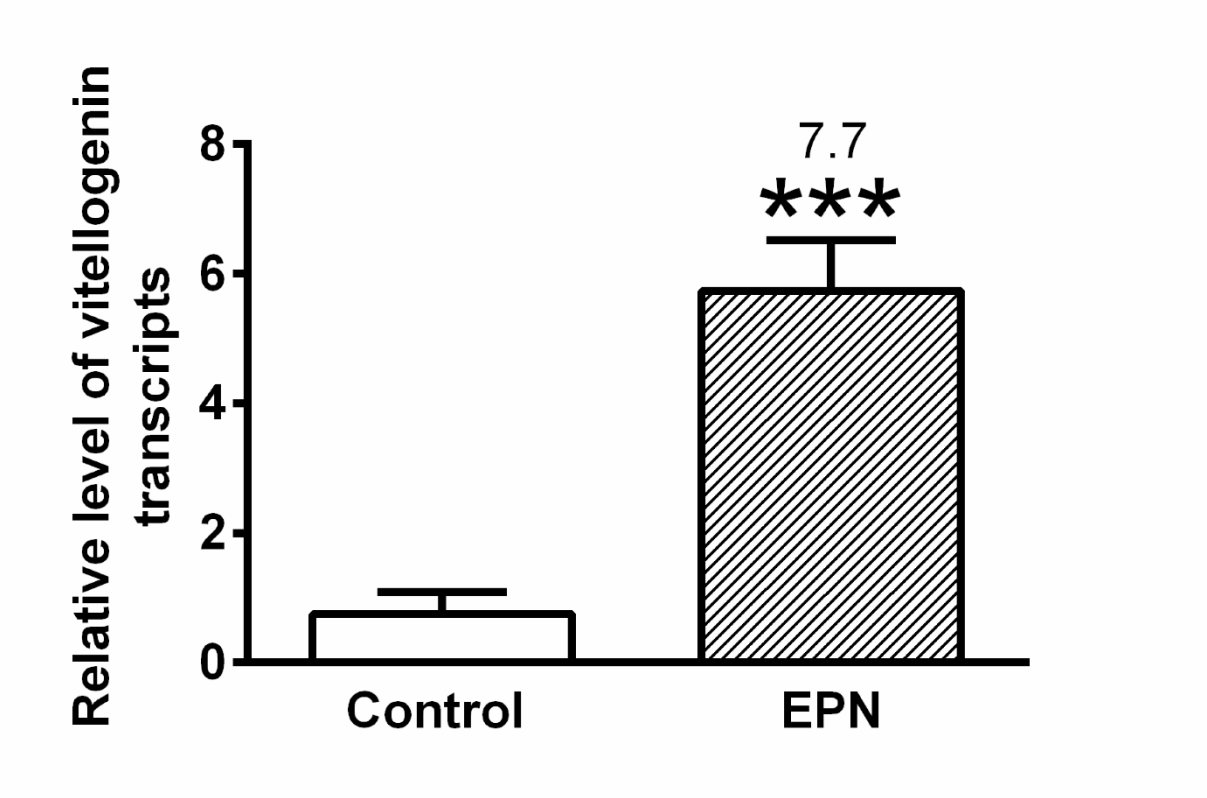
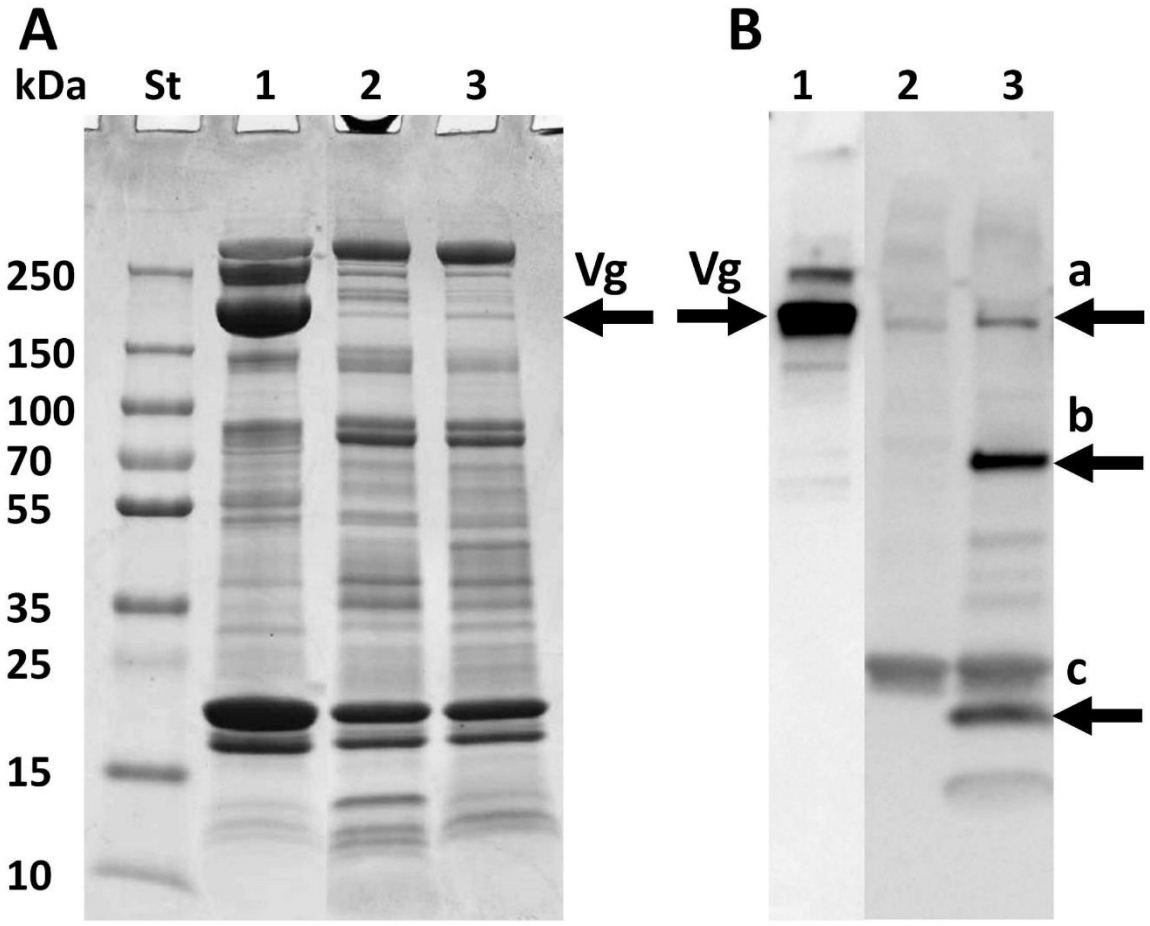


Fig. 1. Relative levels of vitellogenin transcripts in the fat body of 8-day old *P. apterus* males 1 day after the *S. carpocapsae* (EPN) or control treatments (mean ± s.d.). Statistically significant difference between infected and control males at 0.1% level evaluated by Student's t-test is indicated by *** (n=3). The number above the bar represents fold-difference of the vitellogenin transcript levels between the EPN group and control.



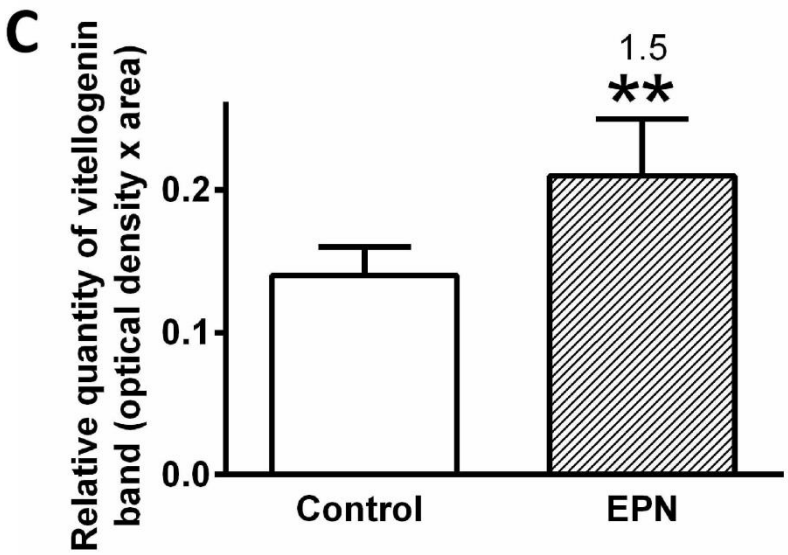


Fig. 2. (A) SDS-PAGE of hemolymph proteins of *P. apterus*: St - MW standards; 1 - control 4-day old females (served for the vitellogenin identification); 2 - 8-day old males 1 day after the control treatment; 3 - 8-day old males 1 day after the *S. carpocapsae* treatment. Vitellogenin (Vg) bands are indicated by the arrow. (B) Western blotting of hemolymph proteins of *P. apterus*. For legends (1-3) see (A). Bands positively reacted with anti-Vg-antibody are indicated by the arrows; estimated MW: a - 180 kDa, b - 65 kDa and C - 24 kDa. (C) Relative level of vitellogenin bands (quantified from corresponding gels that are not shown) in hemolymph of 8-day old *P. apterus* males 1 day after the *S. carpocapsae* (EPN) or control treatments (mean ± s.d.). Statistically significant difference between infected and control groups at 1% level evaluated by Student's t-test is indicated by ** (n=6-7). The number above the bar represents fold-difference of the relative vitellogenin quantities between EPN group and control.

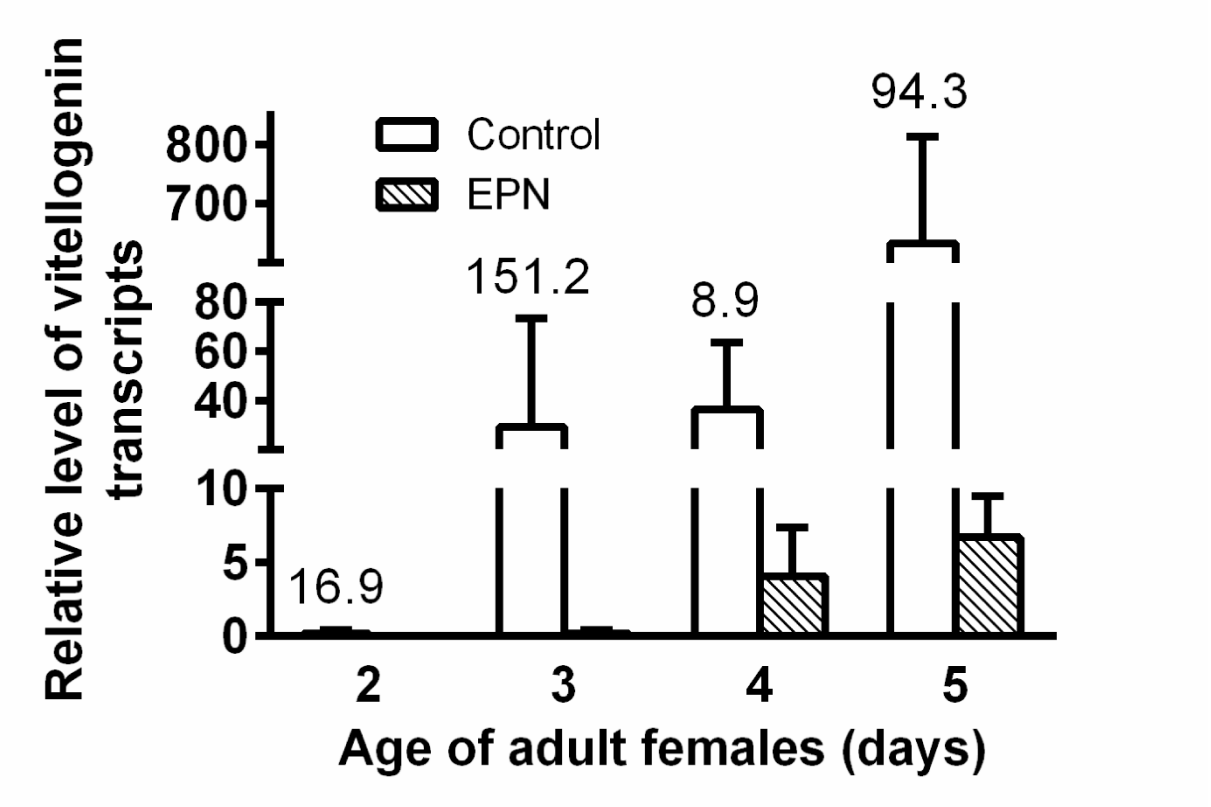


Fig. 3. Relative levels of vitellogenin transcripts in the fat body of 2-, 3-, 4- and 5-day old *P. apterus* females 1 day after the *S. carpocapsae* (EPN) and control treatments (mean ± s.d.). Two-way ANOVA test proved statistically significant difference at 0.1% level between the EPN groups and controls (n=3). The numbers above the bars represent fold-differences of the vitellogenin transcript levels between the EPN group and corresponding control.

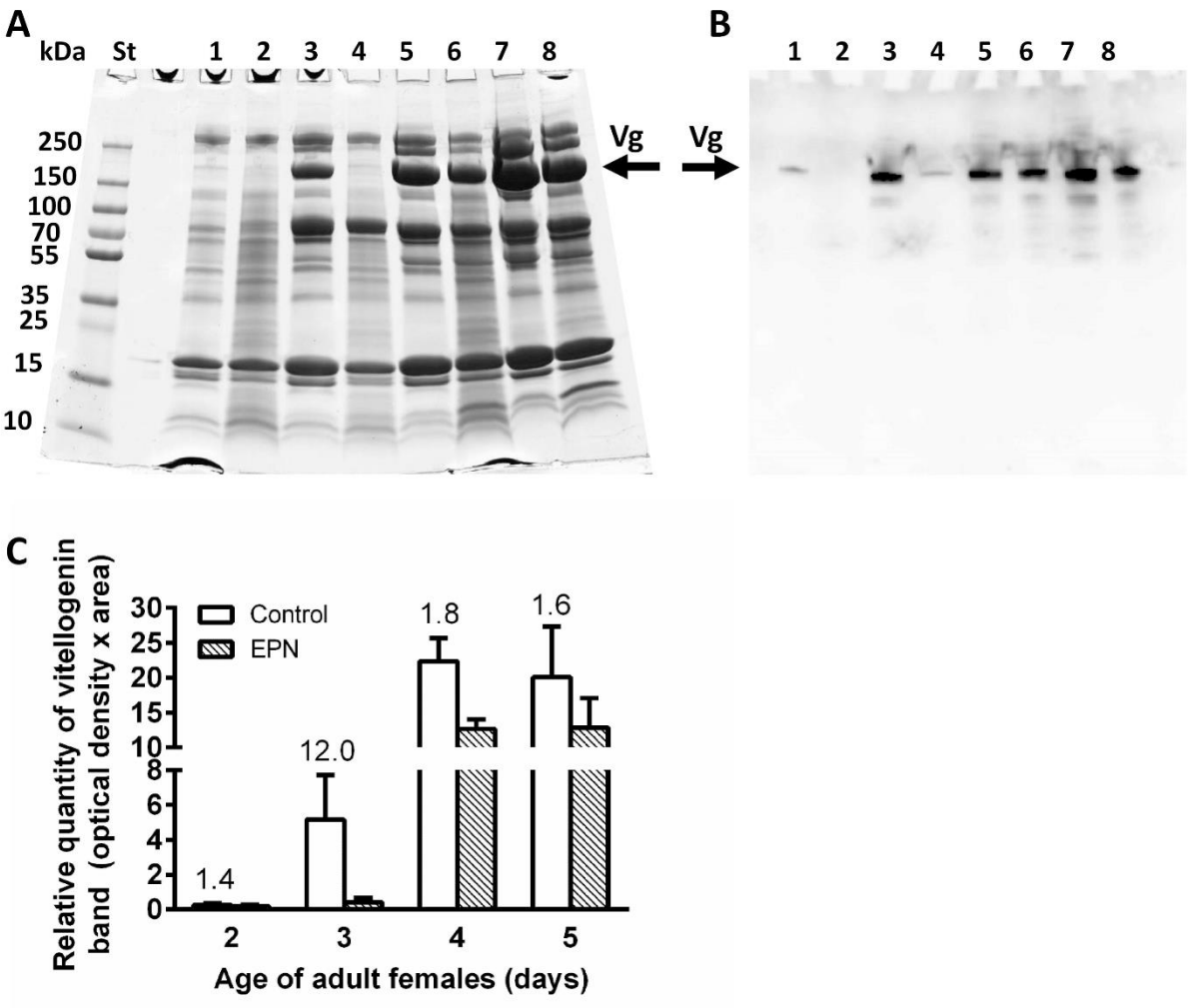


Fig. 4. (A) SDS-PAGE of hemolymph proteins of *P. apterus* females: St - MW standards; 1 + 2 - two-day old females (1 - control, 2 - *S. carpocapsae* treated); 3 + 4 - three-day old females (3 - control, 4 - *S. carpocapsae* treated); 5 + 6 - four-day old females (5 - control, 6 - *S. carpocapsae* treated); 7 + 8 - five-day old females (7 - control, 8 - *S. carpocapsae* treated); all used females were 1 day after the *S. carpocapsae* or control treatments. Vitellogenin (Vg) bands are indicated by the arrow. (B) Western blotting of hemolymph proteins of *P. apterus* females. For legends (1-8) see (A). Bands positively reacted with anti-Vg-antibody are indicated by the arrow. (C) Relative levels of vitellogenin bands (quantified from corresponding gels that are not shown) in hemolymph of 2-5-day old *P. apterus* females 1 day after the *S. carpocapsae* (EPN) or control treatments (mean ± s.d.). Two-way ANOVA test proved statistically significant difference at 0.1% level between the EPN groups and controls (n=6-8). The numbers above the bars represent fold-difference of the relative vitellogenin quantities between EPN groups and corresponding controls.

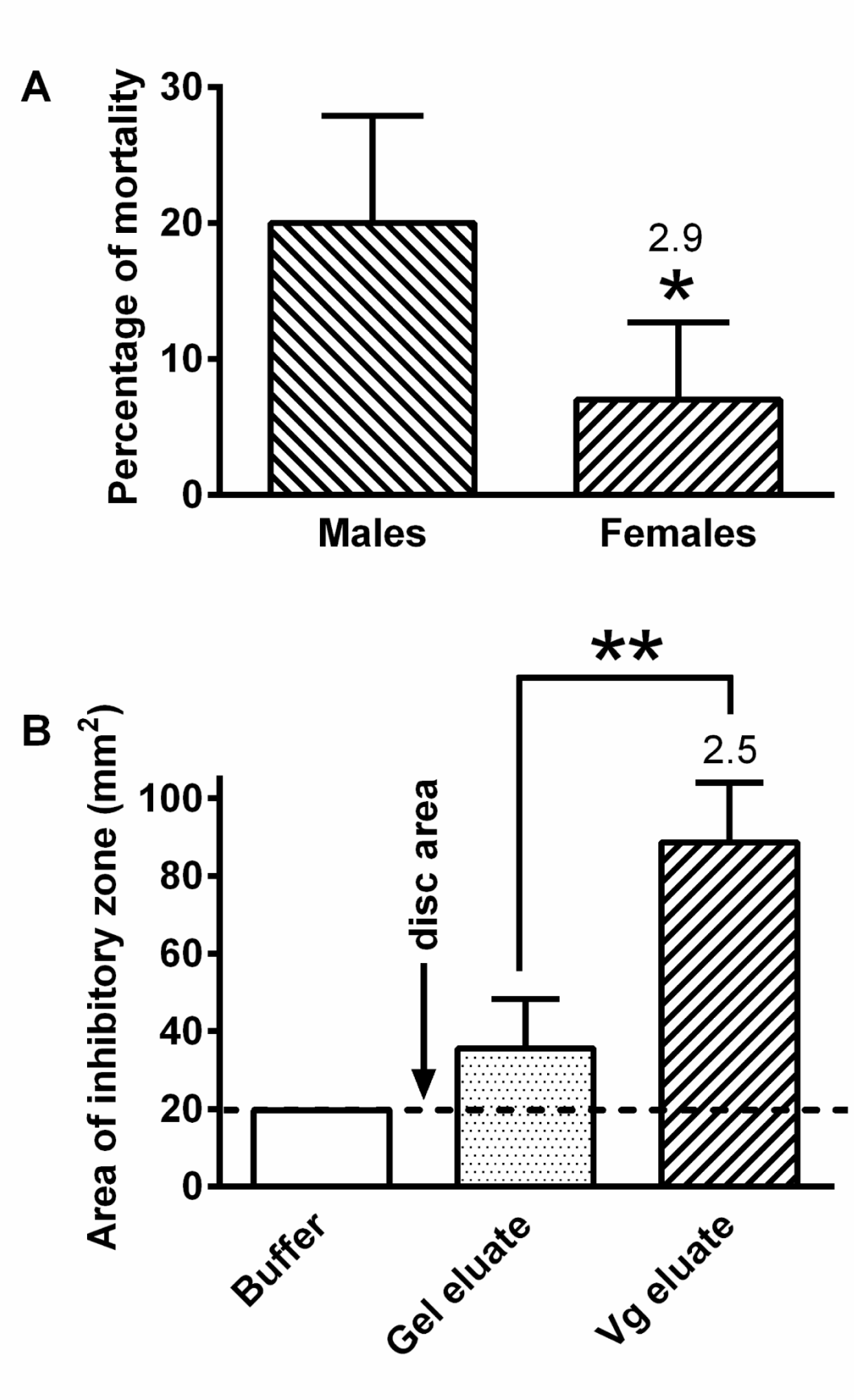


Fig. 5. (A) The effect of *S. carpocapsae* on mortality of *P. apterus* males (7-day old) and females (4-day old) 1 day after the treatment (mean ± s.d.); mortality in controls is not shown being null or negligible. (B) Inhibiting effect of vitellogenin on the growth of the *Xenorhabdus* spp. bacteria tested by the disc diffusion method (mean ± s.d.). Results evaluated 1 day after the vitellogenin application are expressed in areas of inhibiting zones (for details see Material and Methods). Statistically significant differences between the relevant groups at 5% level (A, n=5 groups with 20 adults per each) and 1% level (B, n=5) evaluated by Student's t-test are indicated by * and **, respectively. The numbers above the bars represent fold-difference between the relevant groups.

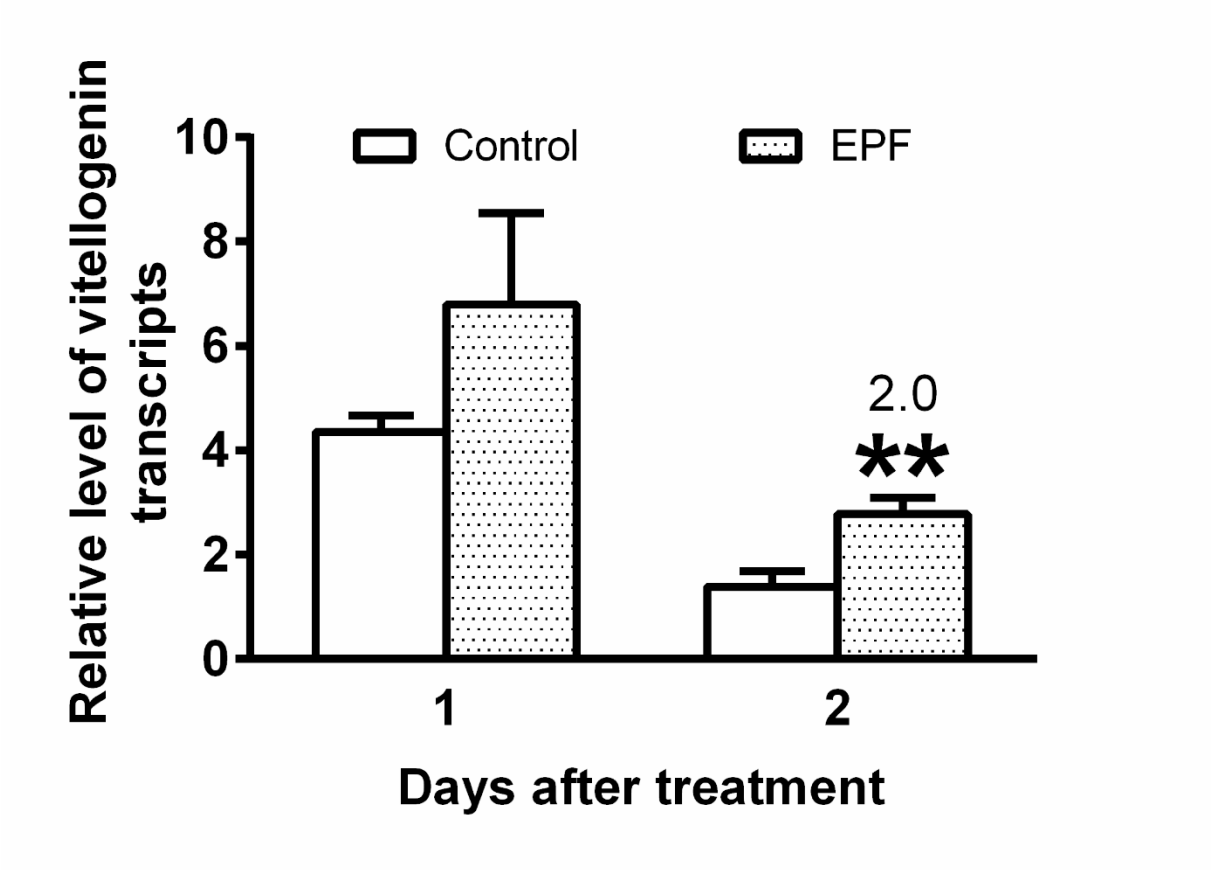


Fig. 6. Relative levels of vitellogenin transcripts in the fat body of *P. apterus* 8- and 9-day old males 1- and 2-day, respectively, after the *I. fumosorosea* (EPF) or control treatments (mean ± s.d.). Statistically significant difference between infected and control males at 1% level evaluated by Student's t-test is indicated by ** (n=3). The number above the bar represents fold-difference of the vitellogenin transcript levels between the EPF group and control.

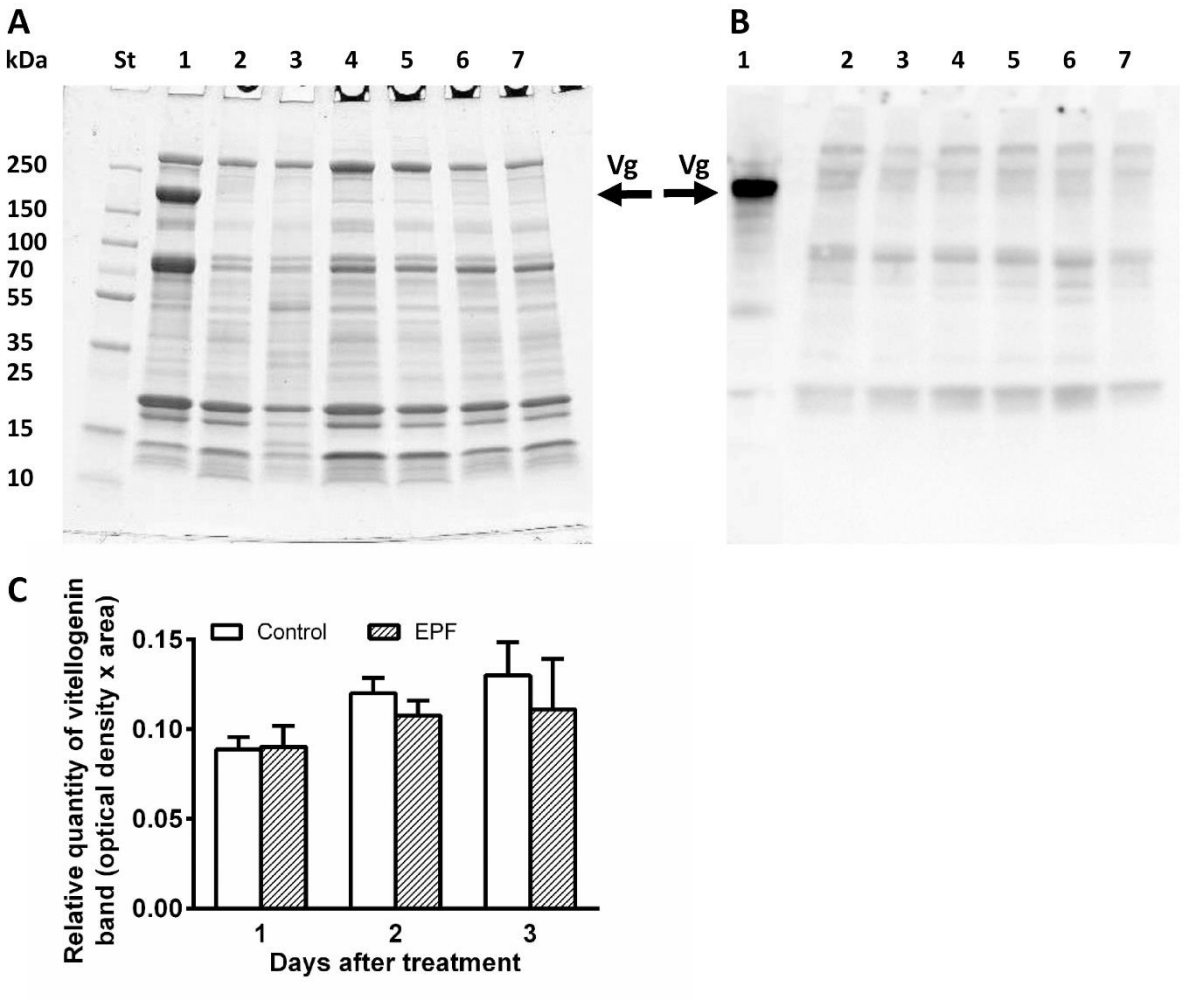


Fig. 7. (A) SDS-PAGE of hemolymph proteins of *P. apterus*: St - MW standards; 1 - control 4-day old females (served for the vitellogenin identification); 2 + 3 - control and *I. fumosorosea*-infected 8-day old males, 1-day after the treatment; 4 + 5 - control and *I. fumosorosea*-treated 9-day old males, 2-day after the treatment; 6 + 7 - control and *I. fumosorosea*-treated 10-day old males, 3-day after the treatment. Vitellogenin (Vg) bands are indicated by the arrow. (B) Western blotting of hemolymph proteins of *P. apterus*. For legends (1-7) see (A). Bands positively reacted with anti-Vg-antibody are indicated by the arrow. (C) Relative levels of vitellogenin bands (quantified from corresponding gels that are not shown) in hemolymph of 8-, 9- and 10-day old *P. apterus* males 1-, 2- and 3-day, respectively, after the *I. fumosorosea* (EPF) or control treatments (mean ± s.d.). No statistically significant difference between infected and control groups evaluated by two-way ANOVA at 5% was recorded (n=6-8).

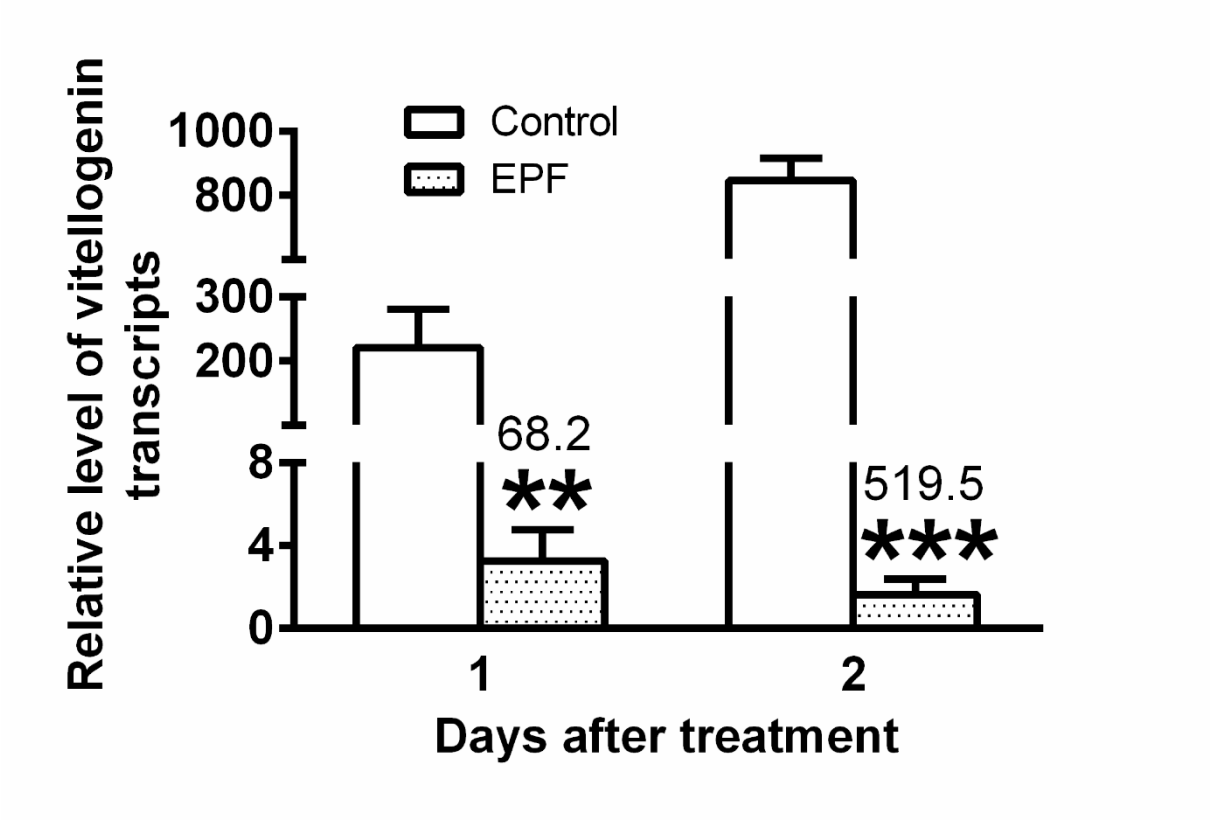


Fig. 8. Relative levels of vitellogenin transcripts in the fat body of 2- and 3-day old *P. apterus* females 1- and 2-day, respectively, after the *I. fumosorosea* (EPF) or control treatments (mean ± s.d.). Statistically significant differences between infected and control females at 1% and 0.1% levels evaluated by Student's t-test are indicated by ** and by ***, respectively (n=3). The numbers above the bars represent fold-difference of the vitellogenin transcript levels between the EPF group and control.

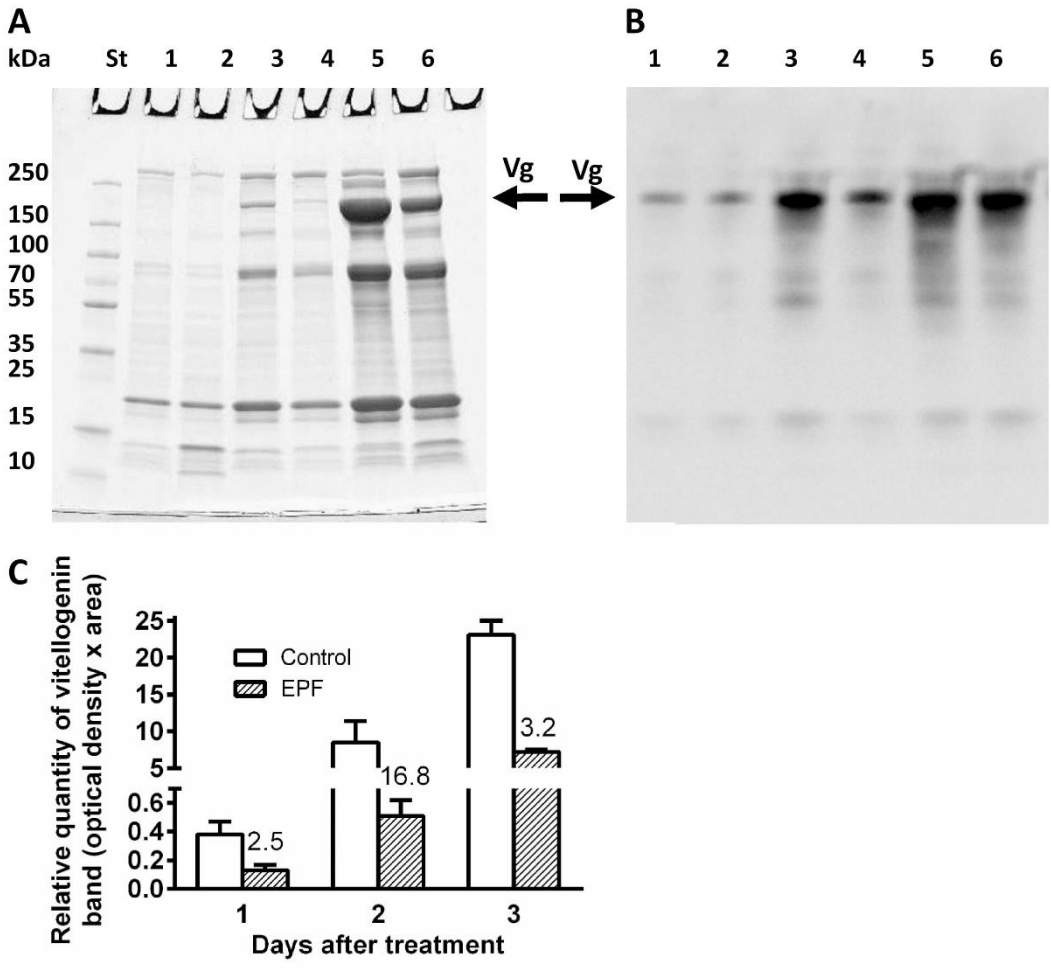


Fig. 9. (A) SDS-PAGE of hemolymph proteins of *P. apterus* females: St - MW standards; 1 + 2 - control and *I. fumosorosea*- infected 2-day old females, 1-day after the treatment; 3 + 4 - control and *I. fumosorosea*- infected 3-day old females, 2-day after the treatment; 5 + 6 - control and *I. fumosorosea*- infected 4-day old females, 3-day after the treatment. Vitellogenin (Vg) bands are indicated by the arrow. (B) Western blotting of hemolymph proteins of *P. apterus* females. For legends (1-6) see (A). Bands positively reacted with anti-Vg-antibody are indicated by the arrow. (C) Relative levels of vitellogenin bands (quantified from corresponding gels that are not shown) in hemolymph of 2-, 3- and 4-day old *P. apterus* females 1-, 2- and 3-day, respectively, after the *I. fumosorosea* (EPF) or control treatments (mean ± s.d.). Two-way ANOVA test proved statistically significant difference at 0.1% level between the EPN groups and controls (n=6-10). The numbers above the bars represent fold-difference of the relative vitellogenin quantities between EPN groups and corresponding controls.

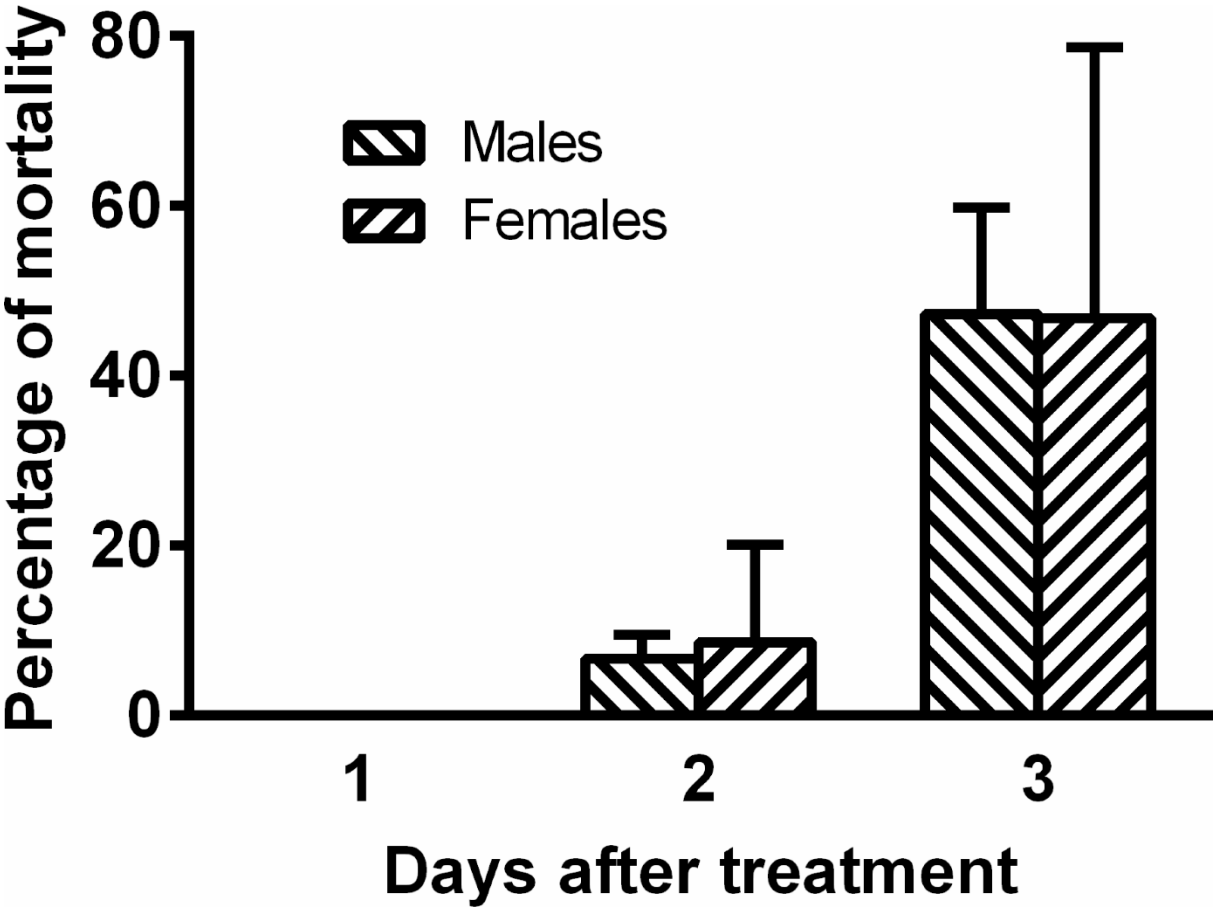


Fig. 10. The effect of *I. fumosorosea* on mortality of *P. apterus* males (7-day old treated) and females (4-day old treated) 1-3-day after the infection (mean ± s.d.); mortality in controls is not shown being null or negligible. No statistically significant difference between males and females evaluated by two-way ANOVA at 5% was recorded (n=11 groups with 20 adults per each).

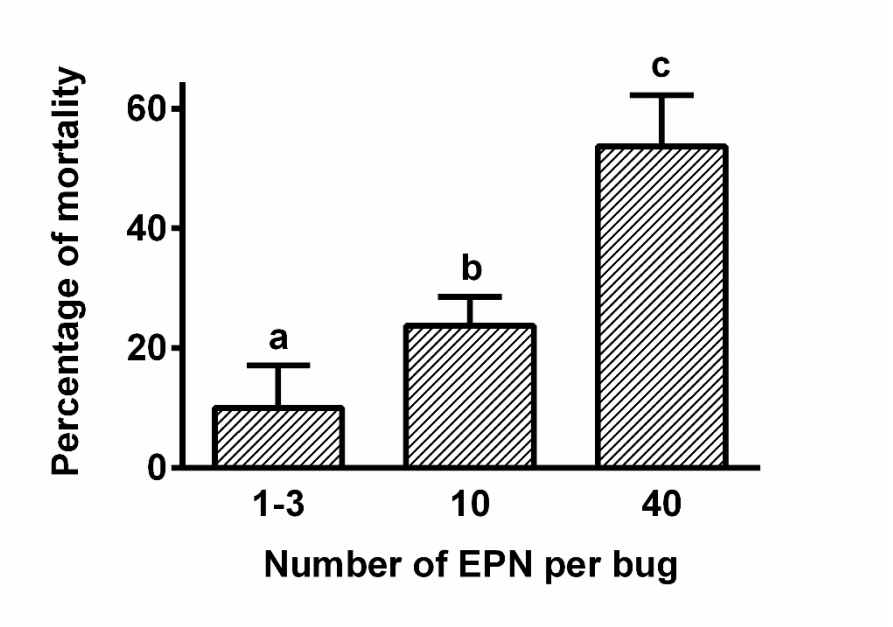
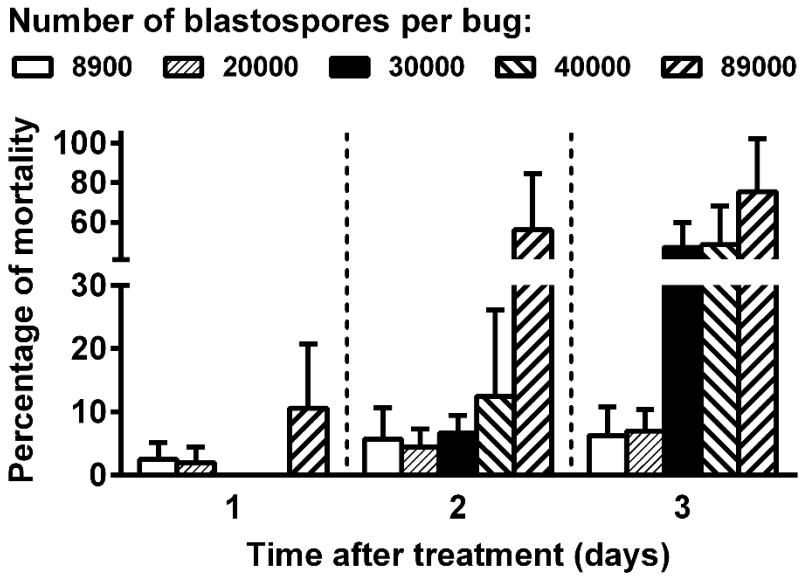


Fig. S1. The effect of various doses of *S. carpocapsae* (EPN) on mortality of *P. apterus* males (8-day old) 1 day after the treatment; mortality in controls is not shown being negligible. Statistically significant difference among infected groups at 5% level evaluated by one-way ANOVA with Tukey's post-test are indicated by different letters (n=6 groups with 20 bugs per each).



2-way ANOVA

Tukey's multiple comparisons test	Mean diff.	95% CI of diff.	Significant?	Summary
8900 vs. 20000	0.3333	-10.25 to 10.92	No	ns
8900 vs. 30000	-13.17	-22.28 to -4.056	Yes	***
8900 vs. 40000	-15.50	-27.55 to -3.448	Yes	**
8900 vs. 89000	-42.50	-51.91 to -33.09	Yes	****
20000 vs. 30000	-13.50	-21.90 to -5.101	Yes	***
20000 vs. 40000	-15.83	-27.36 to -4.310	Yes	**
20000 vs. 89000	-42.83	-51.55 to -34.12	Yes	****
30000 vs. 40000	-2.333	-12.52 to 7.852	No	ns
30000 vs. 89000	-29.33	-36.19 to -22.48	Yes	****
40000 vs. 89000	-27.00	-37.45 to -16.55	Yes	****

Fig. S2. The effect of various doses of *I. fumosorosea* blastospores on mortality of *P. apterus* males (8-, 9- and 10-day old) 1, 2 and 3-day after the treatment; mortality in controls is not shown being null or negligible. Statistics: results of two-way ANOVA with Tukey's post-test are shown in the lower panel (n=8-10 groups with 20 bugs per each).

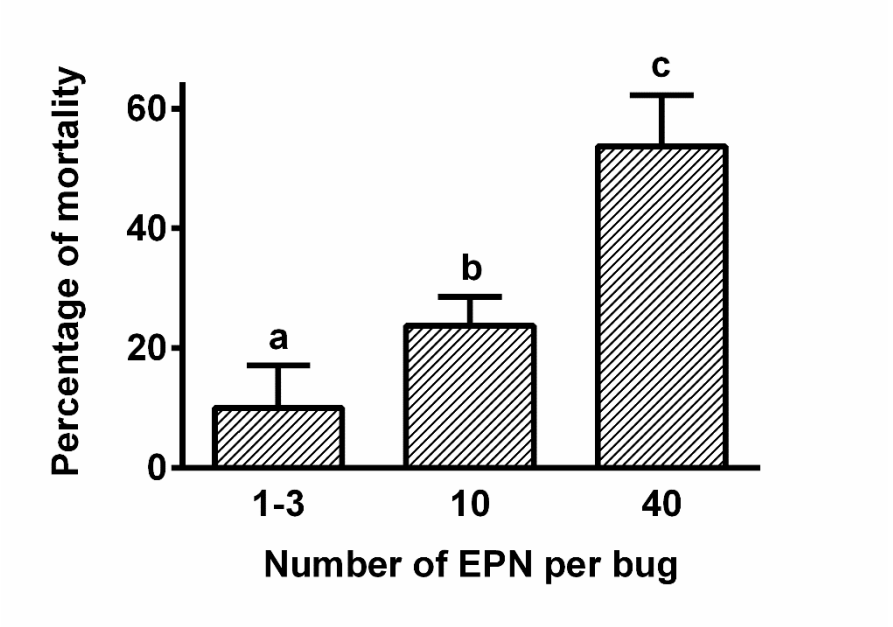
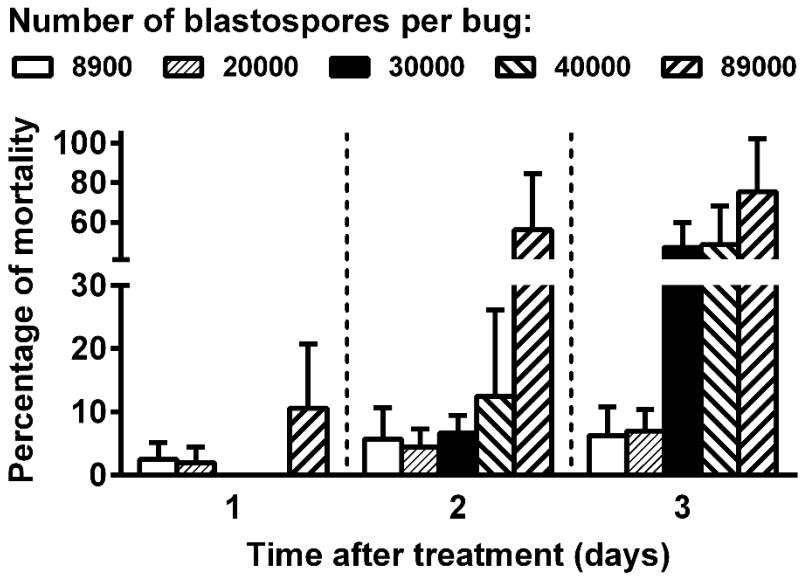


Fig. S1. The effect of various doses of *S. carpocapsae* (EPN) on mortality of *P. apterus* males (8-day old) 1 day after the treatment; mortality in controls is not shown being negligible. Statistically significant difference among infected groups at 5% level evaluated by one-way ANOVA with Tukey's post-test are indicated by different letters (n=6 groups with 20 bugs per each).



2-way ANOVA

Tukey's multiple comparisons test	Mean diff.	95% CI of diff.	Significant?	Summary
8900 vs. 20000	0.3333	-10.25 to 10.92	No	ns
8900 vs. 30000	-13.17	-22.28 to -4.056	Yes	***
8900 vs. 40000	-15.50	-27.55 to -3.448	Yes	**
8900 vs. 89000	-42.50	-51.91 to -33.09	Yes	****
20000 vs. 30000	-13.50	-21.90 to -5.101	Yes	***
20000 vs. 40000	-15.83	-27.36 to -4.310	Yes	**
20000 vs. 89000	-42.83	-51.55 to -34.12	Yes	****
30000 vs. 40000	-2.333	-12.52 to 7.852	No	ns
30000 vs. 89000	-29.33	-36.19 to -22.48	Yes	****
40000 vs. 89000	-27.00	-37.45 to -16.55	Yes	****

Fig. S2. The effect of various doses of *I. fumosorosea* blastospores on mortality of *P. apterus* males (8-, 9- and 10-day old) 1, 2 and 3-day after the treatment; mortality in controls is not shown being null or negligible. Statistics: results of two-way ANOVA with Tukey's post-test are shown in the lower panel (n=8-10 groups with 20 bugs per each).