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RESEARCH REPORT

Entomoparasitic nematodes *Sychnotylenchus sp*. (Anguinidae) on the four-eyed fir bark beetle *Polygraphus proximus*: effects on the host's immunity and its susceptibility to *Beauveria bassiana*

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Abstract

This study for the first time demonstrated evidence of immunosuppressive influence of the entomoparasitic nematode *Sychnotylenchus sp.* (Anguinidae) on the four-eyed fir bark beetle *Polygraphus proximus*. We detected significant decrease of phenoloxidase activity and non-specific esterases and an increase of glutathione-S-transferase rates in nematode-infected beetles. Although infection by the nematode did not cause significant increase of mortality of the four-eyed fir bark beetle, it did enhance its susceptibility to infection by *Beauveria bassiana*. Our observations indicated synergetic effect between nematode and fungus infections. Thus, it was shown that the nematode suppresses immunity of its host only to the extent required for effective implementation of phoresy within the bark beetle.

Key Words: *Polygraphus proximus*; immune suppression; bark beetle culture; nematodes; *Sychnotylenchus*

Introduction

Recent invasion of the four-eyed fir bark beetle, *Polygraphus proximus* (FFBB), a forest pest of Far Eastern origins, into Siberian fir stands (Russia) has had significant ecological and economic consequences (Krivets *et al*., 2015, Kononov *et al*., 2016). The most abundant fungal pathogens of this bark beetle in Siberia are *Beauveria* species (Kerchev *et al*., 2017). However, the complex of natural enemies and pathogens of the FFBB in Siberia remains poorly studied. Nematodes are recognized as one of the major biotic factors affecting populations of aggressive bark beetles (Massey, 1974). Nevertheless, data on interactions of *P. proximus* with roundworms are entirely absent. The roundworms of the genus *Sychnotylenchus* (Anguinidae) are the predominant endoparasitic nematodes (Figs 1a, b) in the West Siberian populations of FFBB (Kerchev and Ryss, 2017 unpublished data) therefore, among other parasites they are of considerable interest.

Nematodes from the genus *Sychnotylenchus* are always associated with bark beetles and their free-living form feeds on fungi growing in the bark beetle frass (Fortunes and Maggenti, 1987). Infection of the insect occurs at the pupal stage, when the nematode juvenile penetrates the host's body cavity through the spiracles. Despite the obvious traits associated with parasitism, these nematodes are not identified as parasites of insects (Fortunes and Maggenti, 1987). The nematodes can be found in the beetle body cavities (Fig. 1c) from pupation of FFBB until oviposition and their presence does not lead to death of the host. Nevertheless, the nematodes infesting the body of the host may instigate an immunomodulatory effect. For endoparasitic nematodes, one of the proven ways to suppress various systems of the host immune response is secretion of immunomodulatory molecules (Cooper and Eleftherianos, 2016). Studies of the interaction of entomopathogenic nematodes with the host's immune system are mainly focused on the effect that the parasite has on the cellular immunity and prophenoloxidase-activating system (proPO cascade) (Cooper and Eleftherianos, 2016). The detoxification pathway, however, remains unclear.

The esterases (EST) and glutathione-S-transferase (GST) are important detoxification enzymes for insects to degrade the toxic substances produced by pathogens. It is known that GST takes part in metabolite removal and protection of tissues from damage by free radicals produced

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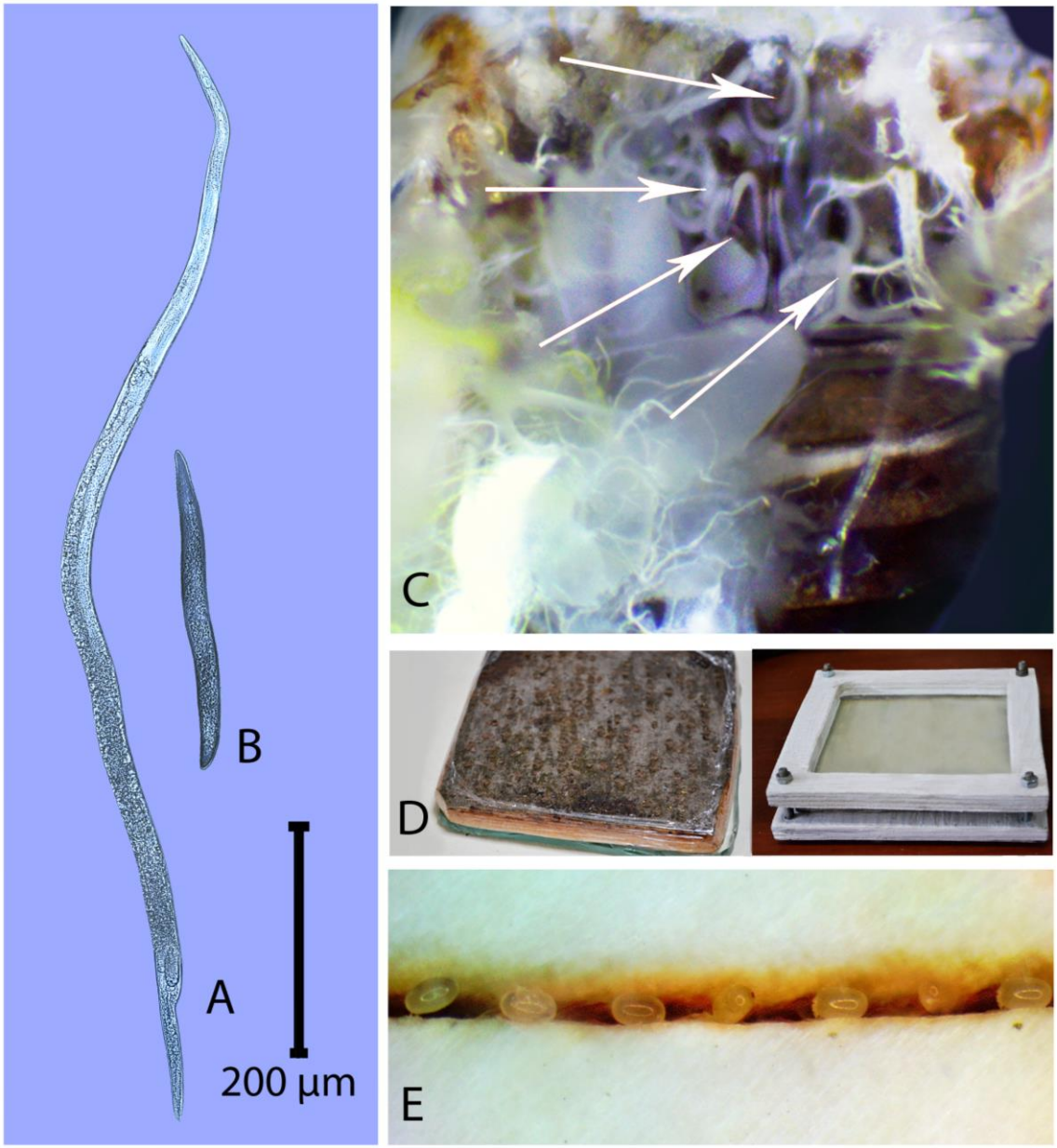

Fig. 1 Rearing of the bark beetle culture and experimental infection by nematodes *Sychnotylenchus sp.* on sandwich plates: A) *Sychnotylenchus sp*. female; B) juvenile stage of nematode from bark beetle hemocel; C) dissection of *Polygraphus proximus* infected by nematodes; D) piece of fir phloem mounted on glass and sandwich plate prepared for rearing of bark beetles; E) washed eggs of bark beetle in the phloem incision.

during pathological processes (Hemingway, 2000; Wu *et al*., 2013). ESTs perform important functions by catabolizing the esters of higher fatty acids, by mobilizing lipids and by degrading inert metabolic esters, including various xenobiotics (Wu *et al*., 2013). Changes in these physiological parameters are associated with insect resistance to entomopathogenic fungi (*e.g.,* Yaroslavtseva *et al*., 2017). A synergistic effect between various pathogenic nematodes and fungi is broadly demonstrated (Ansari *et al*., 2004; Acevedo *et al.,* 2007; Jabbour *et al.,* 2011). In addition, fungi can cause mass death of bark beetles under certain hygrothermal conditions and these are the same conditions that promote mass breeding of the endoparasitic nematodes inhabiting galleries of bark beetles (Shimizu, 2013). However, the influence of nematodes of the genus *Sychnotylenchus* on various parameters of host physiology and susceptibility to fungi has not been studied. Thus, the aim of our work was to quantify the effects of *Sychnotylenchus* sp. infection on FFBB immune and detoxificative system and to assess the impact of nematode infection on the susceptibility of FFBB to infection by the fungus *Beauveria bassiana*.

Materials and Methods

Laboratory populations of the *P. proximus* and endoparasitic nematodes *Sychnotylenchus sp.* were used in all our experiments. A nematode-free line of FFBB was obtained by sterilization of beetle eggs with 3 % solution of hydrogen peroxide. For initiation of nematode infected lines, we designed conditions similar to the natural situation of infection. Larvae of nematodes from the body cavities of infected bark beetles were placed on moistened pieces of filter paper (0.5×0.5 cm) and put near slits in the fir phloem containing sterilized eggs of beetles (10 nematodes per 25 eggs) in sandwich plates (Kerchev, 2014) (Figs 1d, e). The plates were kept at 22 ºC for 60 days. Verification of infection and purity of the lines was performed by dissecting randomly selected beetles under light microscopy. Full details of obtaining nematode infected and nematode free FFBB lines are provided in the electronic supplementary material. Infected and parasite-free *P. proximus* adults were used in experiments on the 55th day after the nematode juveniles had been injected into the fir bark containing FFBB eggs. The presence or absence of melanotic cell capsules around the nematodes was registered using insect dissection and light microscopy. The PO EST and GST activity in the hemolymph were estimated on the adult beetles. All measurements were taken following conventional techniques as follows. For the samples intended for measuring GST and EST activity, phenylthiourea (PTU; 4 µg/ml) was added to each tube containing hemolymph to prevent melanization. PO activity in the hemolymph plasma was determined spectrophotometrically at a wavelength of 490 nm, using solution of dl-dihydroxyphenylalanine (4 mg/ml PB) as a substrate (a modification of the method described by Ashida and Soderhall, 1984). The activity of GST was estimated following Habig *et al*. (1974) and using 2-nitro-5-thiobenzoic acid (DNTB) as a substrate. The concentration of 5-(2, 4-dinitrophenyl)-glutathione produced by the reaction was estimated at a wavelength of 340 nm. EST activity in the samples was determined spectrophotometrically by the Asperen's method (Asperen, 1962) with minor modifications. The concentration of 1-naphthyl produced during the reaction was measured spectrophotometrically at the wavelength of 410 nm. Enzyme activity was measured in units of transmission density (DA) of the incubation mixture during the reaction for each 1 min and 1 mg of protein. The concentration of protein in the hemolymph was determined by the Bradford (1976) method. Bovine serum albumin was used for the calibration curve. For analysis of enzymatic activity 10 replicates (one replicate = 5 beetles) were used. For infection of insects, the culture of *Beauveria bassiana* s.s. SAR-31 from the collection of the Institute of Systematics and Ecology of Animals (ISEA), Siberian Branch, Russian Academy of Sciences, was used. Fungi were cultivated on Sabouraud agar for three weeks. Then conidia were washed from the surface of sporulating areal mycelium and suspended in water with added Tween 20 (0.03 %). Nematode-free and nematode infected beetles were treated with fungi by dipping in a conidia suspension (1×10^7 conidia/mL) for 10 s. The concentrations were counted in a hemocytometer. Fifty insects in each treatment were used. The two control groups of insects, infected with nematodes and nematode-free lines, were dipped in water-Tween solution. Immediately after submersion, the beetles were placed on filter paper to remove extraneous suspension. Then the insects were placed in 60 mm Petri dishes (ten specimens per one dish). Fir bark was returned to the same dishes from which the beetles were previously taken and moistened disks of filter paper were placed to support 100 % RH. After treatment, insects were incubated in the dark at constant temperatures of 18 - 20 °C. Mortality was recorded every day for 15 days. The Mann-Whitney U test was used to estimate differences in enzyme activity in nematode-infected and nematode-free beetles. A log-rank test followed by Holm-Sidak correction was used for analysis of mortality dynamics. The differences between synergistic and additive effects were determined daily by comparing the expected and observed mortality using the χ^2 criterion (Tounou *et al.*, 2008).

Results

We analyzed activity of the PO, EST and GST in FFBB infected by parasitic nematodes of the genus *Sychnotylenchus sp.* A suppressive effect of the nematodes on the immunological status of the infected beetles was evident from our results. Melanotic cell capsules around the nematodes inside the FFBB were not detected which suggests that the immune system of the beetles was not able to recognize nematodes. PO activity of nematode-infected beetles was significantly reduced by 4.7 fold compared to nematode-free beetles ($Z = 2.74$; $p = 0.006$) (Fig. 2A). The activity of non-specific esterases in nematode-infected beetles was also significantly reduced by 3.02 fold ($Z = 3.38$; $p = 0.0007$) (Fig. 2B), while the GTS rate was 2.1 fold higher in nematode-infected beetles ($Z = 2.73$; $p = 0.006$) compared to nematode-free beetles of the control group (Fig. 2C).

Nematode-infected beetles were more susceptible to *Beauveria bassiana* infection compared to nematode-free beetles. Median lethal time (LT_{50}) was 9 ± 0.35 days for nematode-free beetles and 8.00 ± 0.32 days for nematode-infected beetles ($\chi^2 = 13.44$; $p = 0.0002$). A synergistic effect between fungal and nematode infections occurred from 9 to 15 day after treatment with fungus ($\chi^2 > 3.97$; $p < 0.05$) (Fig. 2D). Nematode infection alone causes sublethal effects. However, after 15 days, differences in survival between infected and nematode-free lines were not significant ($\chi^2 = 3.41$; $p = 0.06$) data not shown.

Discussion

The absence of the capsule around nematodes and the decrease of the phenoloxidase activity

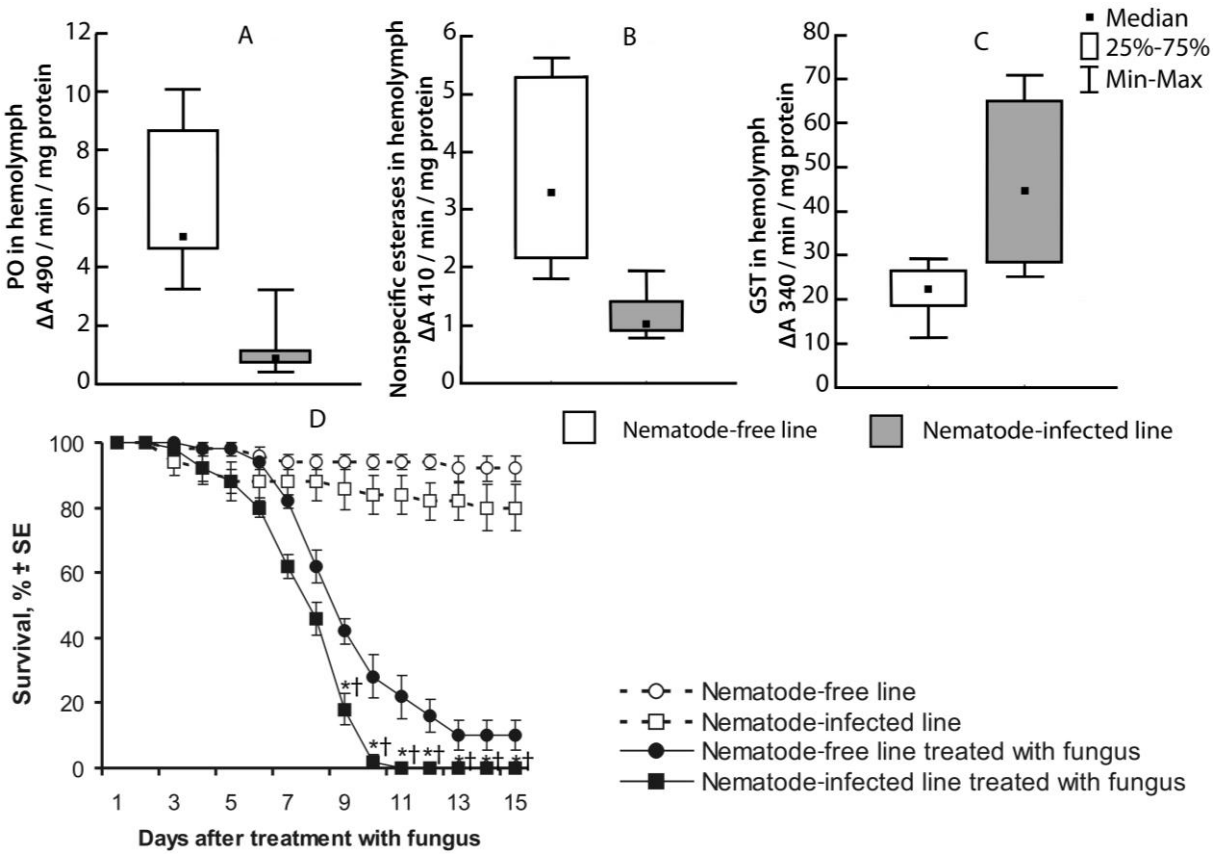

Fig. 2 Activity of immune and detoxificative enzymes in hemolymph of *Polygraphus proximus* adults which were infected and non-infected with nematode *Sychnotylenchus sp.*, and influence of nematode infection on susceptibility to *Beauveria bassiana*. A) phenoloxidase activity, B) non-specific esterases activity, C) glutathione-S-transferase activity, D) survival dynamics after *B. bassiana* treatment. † - significant differences between the lines infected and non-infected with nematode ($Z > 2.73$; $p < 0.006$), * - synergistic effect between nematode and fungus ($\chi^2 > 3.97$; $p < 0.05$).

suggest that an active mechanism functions in these immune reactions inhibited. The immediate response of host beetles to nematodes is encapsulation, accompanied by the release of a toxic reactive oxygen species (ROS) formed during melanization that leads to destruction of the pathogens (Dowds and Peters, 2002). There are a number of intermediates similar to ROS linked with the melanotic cascade (quinones, hydrogen peroxide, O-semiquinone radicals, etc.) that are cytotoxic and destroy pathogenic microorganisms (Nappi and Ottaviani, 2000; Komarov *et al*., 2009). On the other hand, active oxygen radicals are quite toxic to the host and subsequently detoxified by enzymes to prevent oxidative stress. Reduced activity of PO is the most common method to expose the parasite to the host's immune response. The inhibition of PO activity may be due to secretion of immunosuppressive molecules into the hemocel of the insect by the parasite itself, and/or by its symbiont microorganisms (Cooper and Eleftherianos, 2016). *Steinernema carpocapsae* secretes chymotrypsin proteases inhibiting the prophenoloxidase cascade to avoid encapsulation in the hemocel of *Galleria mellonella* (Balasubramanian *et al*., 2010). The nematode species *S. feltiae* induces prophenoloxidase inactivation in *G. mellonella* larvae by interaction of nematode cuticular components with the prophenoloxidase activating LPS-binding proteins (Brivio *et al*., 2002). The main detoxification enzymes in invertebrates are monooxygenases, EST and GST. EST participate in many biological functions and begin the metabolism of xenobiotics, lipids, acetylcholine and juvenile hormone (Kumar *et al*., 2003; Lozinskaya *et al*., 2004; Li *et al*., 2007). Our observed effect of *Sychnotylenchus sp.* on the detoxification enzymes of FFBB is similar to the results of Wu *et al*. (2013) and Li *et al. (*2016). Authors detected significantly dose-dependent enhancement of the GST activity in host larvae was detected along with a decrease of the carboxylesterase activity on the systems of *G. mellonella* larvae when infested with the entomopathogenic nematode *Heterorhabditis beicherriana n. sp.* (Rhabditida: Heterorhabditidae) and on *Tenebrio molitor* (Coleoptera: Tenebrionidae) larvae when infested with *H.*

beicherriana. We observed an increase in the GST activity and that can be both a result of the general depression of the PO activity by toxic or mechanical damage to host cells and tissues by the nematodes or an adaptive response to neutralize ROS induced by oxidative stress.

The decrease of the EST activity in hemolymph is probably because ones besides detoxification take a direct part in lot of physiological processes in insects. Many physiological processes of insects are resource consuming and can go against the interests of the parasite; as a result, total count of enzymes can be depleted by nematode.

Depression in the functioning of the host's immune system induced by *Sychnotylenchus sp*. contributes to an increasing susceptibility for entomopathogenic fungi. It has been confirmed that ascomycetes as the generalists are adapted to infect insects weakened by various ecological factors (Boomsma *et al.,* 2014) and in this case with immune systems suppressed by nematode infestation. From an ecological point of view, the FFBB- *Sychnotylenchus sp*. system is one of the examples of a stable host-parasite relationship, in which the nematode suppresses the host's immunity only as much as is required for the effective implementation of phoresy within the host.

This is the first study to determine an influence of nematodes from the genus *Sychnotylenchus* on its host's physiological system. Nematode infection causes sub lethal effects on *P. proximus* adults, but significantly modulates immune and detoxificative systems that lead to increase in susceptibility to fungal infection.

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References

Acevedo JPM, Samuels RI, Ribeiro MI, Dolinski C. Interactions between isolates of the entomopathogenic fungus *Metarhizium anisopliae* and the entomopathogenic nematode *Heterorhabditis bacteriophora* JPM4 during infection of the sugar cane borer *Diatraea saccharalis* (Lepidoptera: Pyralidae). J. Invertebr. Pathol. 96: 187-192, 2007.

Ansari MA, Tirry L, Moens M. Interaction between Metarhizium anisopliae CLO 53 and entomopathogenic nematodes for the control of *Hoplia philanthus*. Biol. Control 31: 172-180, 2004.

Asperen K. Van A study of housefly esterases by means of a sensitive colorimetric method. J. Insect Physiol. 8: 401-414, 1962.

Balasubramanian N, Toubarro D, Simões N. Biochemical study and in vitro insect immune suppression by a trypsin-like secreted protease from the nematode Steinernema carpocapsae. Parasite Immunol. 32: 165-175, 2010.

Boomsma JJ, Jensen A B, Meyling NV, Eilenberg J. Evolutionary Interaction Networks of Insect Pathogenic Fungi. Annu. Rev. Entomol. 59: 467-485, 2014.

Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein dye binding. Anal. Biochem. 72: 248-254, 1976.

Brivio MF, Mastore M, Pagani M. Parasite-host relationship: a lesson from a professional killer. Inv. Surv. J. 2: 41-53, 2005.

Brivio MF, Pagani M, Restelli S. Immune suppression of *Galleria mellonella* (Insecta, Lepidoptera) humoral defenses induced by *Steinernema feltiae* (Nematoda, Rhabditida): involvement of the parasite cuticle. Exp. Parasitol. 101: 149-156, 2002.

Cooper D, Eleftherianos I. Parasitic nematode immunomodulatory strategies: recent advances and perspectives. Pathogens 5: 58, 2016.

Dowds BCA, Peters A. Virulence mechanisms. In Entomopathogenic Nematodes, Gaugler R (Ed.), CABI: Wallingford, UK, pp 79-98, 2002.

Dubovskiy IM, Slyamova ND, Kryukov VYu, Yaroslavtseva ON, Levchenko MV, Belgibaeva AB, *et al.* The activity of nonspecific esterases and glutathione-S-transferase in *Locusta migratoria* larvae infected with the fungus *Metarhizium anisopliae* (Ascomycota, Hypocreales). Entomol. Rev. 92: 27-32, 2012.

Fortuner R, Maggenti AR. A reappraisal of Tylenchina (Nemata). The family Anguinidae Nicoll, 1935 (1926). Rev. Nematol. 10: 163-176, 1987.

Habig WH, Pabst MJ, Jakoby WB. Glutathione-S-transferases. J. Biol. Chem. 249: 7130-7139, 1974.

Hemingway J. The molecular basis of two contrasting metabolic mechanisms of insecticide resistance. Insect Biochem. Mol. Biol. 30:1009-1015, 2000.

Jabbour R, Crowder DW, Aultman EA, Snyder WE. Entomopathogen biodiversity increases host mortality. Biol. Control 59: 277-283, 2011.

Kerchev IA, Kryukov VYu, Yaroslavtseva ON, Polovinko GP, Tokarev YuS, Glupov VV. The first data on fungal pathogens (Ascomycota, Hypocreales) in the invasive populations of four-eyed fir bark beetle *Polygraphus proximus* Blandf. Russian J. Biol. Invasions 8: 34-40, 2017.

Kerchev IA. On monogyny of the four-eyed fir bark beetle *Polygraphus proximus* Blandf. (Coleoptera, Curculionidae: Scolytinae) and its reproductive behavior. Entomol. Rev. 94: 1059-1066, 2014.

Komarov DA, Ryazanova AD, Slepneva IA, Khramtsov VV, Dubovskiy IM, Glupov VV. Pathogen-targeted hydroxyl radical generation during melanization in insect hemolymph: EPR study of probable cytotoxicity mechanisms. Appl. Magnetic Resonance, 35: 495-501, 2009.

Kononov A, Ustyantsev K, Blinov A, Fet V, Baranchikov YuN. Genetic diversity of aboriginal and invasive populations of four-eyed fir bark beetle *Polygraphus proximus* Blandford (Coleoptera, Curculionidae, Scolytinae). Agr. Forest. Entomol., 2016. DOI: 10.1111/afe.12161.

Krivets SA, Bisirova EM, Kerchev IA, Pats, EN, Chernova NA. Transformation of taiga

ecosystems in the Western Siberian invasion focus of four-eyed fir bark beetle *Polygraphus proximus* Blandford (Coleoptera: Curculionidae, Scolytinae). Russian J. Biol. Invasions 6: 94-108, 2015.

Kumar S, Christophides GK, Cantera R, Charles B, Han YS, Meister S, *et al.* The role of reactive oxygen species on Plasmodium melanotic encapsulation in *Anopheles gambiae*. Proc. Natl. Acad. Sci. USA 100: 14139-1414, 2003.

Li Y, Lopez P, Durand P, Ouazzani J, Badet B, Badet-Denisot MA. An enzyme-coupled assay for amidotransferase activity of glucosamine-6-phosphate synthase. Anal. Biochem. 370: 142-146, 2007.

Lozinskaya YaL, Slepneva IA, Khramtsov VV, Glupov VV. Change of the antioxidant status and free radicals production in hemolymph of *Galleria mellonella* larvae in microsporidiosis. J. Evol. Biochem. Physiol. 40: 119-125, 2004.

Massey CL. Biology and taxonomy of nematode parasites and associates of bark beetles in the United States. Forest Service, US Dept. of Agriculture, Washington, DC, Washington, 1974.

Nappi A, Ottaviani E. Cytotoxicity and cytotoxic molecules in invertebrates. BioEssays 22: 469-480, 2000.

Tounou, AK, Kooyman C, Douro-Kpindou OK, Poehling HM. Interaction between *Paranosema locustae* and *Metarhizium anisopliae* var. acridum, two pathogens of the desert locust, *Schistocerca gregaria* under laboratory conditions. J. Invertebr. Pathol. 97: 203-210, 2008.

Wu H-D, Liu Q-Z, Li X-Y, Wang Y-L, Zhang H. Activities of four enzymes in *Galleria mellonella* larvae infected with entomopathogenic nematode *Heterorhabditis beicherriana n. sp*. Afr. J. Agric. Res. 8: 3245-3250, 2013.

Xingyue Li, Qizhi Liu, Edwin E. Lewis, Eustachio Tarasco Activity changes of antioxidant and detoxifying enzymes in *Tenebrio molitor* (Coleoptera: Tenebrionidae) larvae infectedby the entomopathogenic nematode *Heterorhabditis beicherriana* (Rhabditida: Heterorhabditidae). Parasitol. Res., 2016. DOI 10.1007/s00436-016-5235-7.

Yaroslavtseva ON, Dubovskiy IM, Khodyrev VP, Duisembekov BA, Kryukov VYu, Glupov VV. Immunological mechanisms of synergy between fungus *Metarhizium robertsii* and bacteria *Bacillus thuringiensis* ssp. morrisoni on Colorado potato beetle larvae. J. Insect Physiol. 96: 14-20, 2017.

Supplementary material

Obtaining nematode-free and nematode-infected lines of four eyed fir bark beetles

The laboratory culture of four eyed fir bark beetles free from nematode parasites was produced from natural population from West Siberia (Tomsk Region, near the settlement of Zavarzino, 56° 27'45" N, 85° 05'25" E). In this stand, the infection of beetles by *Sychnotylenchus sp*. reached 97 %. Collected beetles were put in plastic containers on the freshly cut logs of Siberian fir *Abies sibirica* Ledeb. On the 14th day after introduction of parent beetles, bark was removed from the logs. Only unhatched eggs from the maternal galleries were used for rearing insect culture. To remove eggs or larvae of nematodes from the surface of the chorion, bark beetle eggs were put in a sieve with a cell size of 0.2 mm, and then placed for 30 sec. in 3 % solution of hydrogen peroxide followed by 10 min. washing in distilled water. This procedure was repeated twice. After washing, the eggs in batches of 25 pcs were placed on freshly-peeled fir phloem (15×15 cm) into 2.5 cm long incisions. The phloem tissue containing eggs was mounted on sandwich plates (Kerchev, 2015). To prevent desiccation, the sides were covered with parafilm. A total of six sandwich plates were prepared each containing 150 bark beetle eggs free from nematodes. After 5 days, half of the plates were injected with nematode juveniles from the hemocoel of native *P. proximus* beetles. After preliminary identification of a taxonomical group of nematodes under a light microscope, nematode larvae, along with a drop of distilled water, were transferred from the slide in groups of 10 pcs onto a 0.5×0.5 cm filter paper, which was placed next to phloem with eggs on sandwich plates. Plates with insects were kept for 60 days at 22 °C and relative humidity of 80 %. The presence of nematodes in larval galleries on sandwich plates and beginning of invasion of juveniles into bark beetles pupae was checked through glass. The infection of beetles was assayed on 20 randomly selected beetles per line. All insects in the nematode-infected line were invaded by larvae of *Sychnotylenchus sp*. and the intensity of infection was 18.2 ± 6.4 (Mean ± SD) per insect.