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Short Communication

Bacterial and fungal infections induce bursts of dopamine in the haemolymph of the Colorado potato beetle *Leptinotarsa decemlineata* and greater wax moth *Galleria mellonella*

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ABSTRACT

Dopamine (DA) is known as a hormone neurotrasnmitter molecule involved in several stress reactions in both vertebrates and invertebrates. Following infections with the fungi *Metarhizium robertsii* or *Beauveria bassiana* and the bacterium *Bacillus thuringiensis*, dopamine the concentration was measured at different time points in the haemolymph of the Colorado potato beetle, *Leptinotarsa decemlineata* and the larvae of the greater wax moth *Galleria mellonella*. The infection with *M. robertsii* increased (4 to 12-fold) DA concentrations in the haemolymph of the potato beetle larvae and the oral infection by *B. thuringiensis* also lead to a 30 and 45-fold increase. During infection of the greater wax moth larvae with *Beauveria bassiana* and *B. thuringiensis* DA increased 4 to 20-fold and about 2 to 2,5-fold respectively, compared to non-infected insects. The relative DA concentrations varied between the two insects and depended on the pathogens and post infection time.

1. Introduction

Bio-insecticides based on the bacteria *Bacillus thuringiensis* and fungi *Beauveria* and *Metarhizium* are used to control many pest insects (Lacey et al., 2015). The penetration by the entomopathogenic microorganisms in insects leads to the activation of various defence reactions that may result in the delay or elimination of an infection. Phagocytosis, encapsulation, synthesis of the antimicrobial proteins and prophenoloxidase (proPO) cascades have important roles in the resistance of insects to the entomopathogenic fungi and bacteria (Grizanova et al., 2014; reviewed by Butt et al. (2016) and Wojda (2017). Stress-mediated reactions also play a key role during the development of fungal and bacterial infections (Feder and Hofmann, 1999; Taszlow and Wojda, 2015). It is known the elevated level of heat shock proteins (HSP) in organisms indicates a stress condition and means that stress reactions will be actively developed (Lindquist, 1986; Wojda and Taszlow, 2013; Grizanova et al., 2014). Dubovskiy et al. (2013a) have shown that expression Hsp90 has increased after insect infection by the entomopathogenic fungi *Beauveria bassiana*. Evidently, the infectious process leads to the activation of the various defence systems in insect organisms and promotes the production of signal molecules that indicate stress development.

Neuroendocrine stress reaction is a universal and effective instrument of insect protection from stress. The stress reaction of insects is a complex process involving endocrine reactions in organisms (Rauschenbach et al., 1987). Various hormones are involved in stress reactions of insects, including, biogenic amines (dopamine, octopamine, serotonin) and gonadotropins (ecdysteroids and juvenile hormone). The role of neurohormone dopamine (DA) during the infection process is poorly understood. DA takes part in cuticle formation (Nappi and Christensen, 2005; Watanabe et al., 2013) and influences the synthesis of other hormones (Pendleton et al., 2002). Additionally, DA is involved in behavioural reactions, reproduction and motorial activity (Kume et al., 2005). It has been shown that the different ecological stressors and the parasitoid wasp (*Cotesia kariyai)* could increase the concentration of DA (Noguchi et al., 1995; Hirashima et al., 2000; Neckameyer and Weinstein, 2005). It has been shown that DA concentration influences grooming intensity, which is the one of the protection mechanisms insects use against fungi (Zhukovskaya et al., 2013). Moreover, the DA is an integral part of the proPO cascade during melanic capsule formation (Andersen, 2010; Dubovskiy et al., 2016b) and DA is one the signal moieties responsible for mediating phagocytosis by insect haemocytes (Wu et al., 2015). Thus, the DA is involved in both the behavioural and physiological defence reactions against

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infections.

The Colorado potato beetle (*Leptinotarsa decemlineata* Say.) (CPB) is one of the most dangerous pests in North America, Eurasia and Africa. CPB can acclimate and propagate in a variety of worldwide habitats due to their active migration capacity, high plasticity and broad spectrum of intraspecific polymorphism (Weber, 2003). The greater wax moth (*Galleria mellonella* (L.)) is considered a pest of beehives and a popular model species used for immune and physiological research, as well as for testing infections of insects and mammals (Wojda, 2017).

This research presents one of the first evidence of the DA concentration increase in the haemolymph of the Colorado potato beetle and the greater wax moth larvae during fungal and bacterial infections.

2. Materials and methods

Colorado potato beetle larvae were collected from farmer plantations of potato *Solanum tuberosum* in the Novosibirsk region, where there were no applications of chemical insecticides. Collected insects were maintained under laboratory conditions at LD 12:12 and at 25 °C. The larvae were kept in 300-ml plastic air containers (10 insects per container). The larvae were fed fresh cut shoots of potato *S. tuberosum*. The wax moths *G. mellonella* were reared in strict isolation at 28 °C, 60% relative humidity, 12-h photoperiod, and fed on artificial medium (AM) containing 22.5% corn meal, 12.5% honey, 12.5% glycerol, 12.5% beeswax, 10% wheat flour, 12.5% milk solids, 5% yeast and 12.5% water. For experiments, we used the fourth instars CPB larvae, which were no older than 10 h after moulting and the four instar wax moth larvae.

The fungus *Metarhizium robertsii* (isolate Mak-1) (Mr) and bacterium *Bacillus thuringiensis ssp. morrisoni var. thuringiensis* (strain 2495) (Bt) were used to infect the CPB larvae. *Beauveria bassiana* (isolate Sar-31) (Bb) and bacterium *Bacillus thuringiensis ssp. morrisoni var. thuringiensis* (strain 2495) (Bt) were used to infect the wax moth larvae. All strains of entomopathogenic microorganisms were provided by the ISEA collection. Conidia of Bb and Mr were grown on double autoclaved millet (Kryukov et al., 2009). Spores and crystals of bacterium were grown on meat peptone agar. LD50 doses were used for insect infection (4×10^6 conidia/ml of Mr, 5×10^8 crystals and spores/ml of Bt and 1×10^6 conidia/ml of Bb).

Topical application was used for insect infection with Bb and Mr. and was performed by a single dipping (10 s) of insects into water-Tween-20 (0.03%) suspensions of fungal conidia. Insects of the control group were treated with water-Tween-20 (0.03%) solution. Oral inoculation was used for CPB larvae infection with Bt by dipping (10 s) potato leaves into suspension of the bacterial spore-crystal mix in saline solution (NaCl 0.9%). The control insect group was feed leaves treated with saline solution (NaCl 0.9%). Oral inoculation was used for wax moth larvae infection with Bt by adding of 1 ml of the bacterial spore-crystal mix into 3 g of artificial medium. The control insect group was feed on artificial medium mixed only with saline solution.

The DA concentration was measured using a slightly modified method of Gruntenko et al. (2005). The alive larvae were surface sterilised by submersion in 95% ethanol. The haemolymph of the insects was collected in 0.2 M HClO4 and pooled from 3 CPB larvae (16.6 µl to each in one tube) 24, 48 and 72 h post-infection and from 5 larvae of wax moths (10 µl to each in one tube) 24, 72 and 120 h post-infection. A haemolymph sample was admixed with 0.2 M HClO4 at the ratio 1:1. The samples ware incubated at 28 °C and 600 rpm for 10 min. Then, the samples were centrifuged at 4 °C within 10 min at 10000*g*. The supernatant was filtered through a syringe nylon filter, and a 10-µl aliquot of the supernatant was directly analysed by HPLC-ECD (electrochemical detection). Chromatography was carried out in a C18 reverse-phase column (Zorbax SB-C18 4.6 mm × 250 mm, 5 µm average particle size, Agilent, USA) using a 1260 HPLC-ECD system (Agilent Technologies Inc., Santa Clara, California) with a quaternary pump, vacuum degasser, autosampler, thermostatically controlled column compartment and a coulometric electrochemical detection system (ESA 5010A, using potential 300 mV, Bedford, MA). The flow rate was maintained at 1 ml/min, and the mobile phase consisted of a 0.026 M buffer (pH 3.0) (KH_2PO_4), 1-Octanesulfonic acid (200 mg/l) as an ion-pair reagent and 10% acetonitrile. The concentration of DA was calculated by comparing the peak area between the sample and standard (Dopamine hydrochloride, Sigma-Aldrich). At least 10 samples were examined per treatment and per time point. The experiments ware repeated independently three times.

The obtained data are presented as the average ± standard error. Data were not normally distributed (Agostino-Pearson omnibus test) due to biological variation; therefore, we used non-parametric analysis (one-way non-parametric ANOVA; Kruskal-Wallis with Dunn's post-test) (GraphPad Prism v4.0 (GraphPad Software, USA).

3. Results and discussion

The mortality of CPB larvae was at 0%, 10%, 19.6% at 24, 48 and 72 h respectively following infection with *Bt* after the treatment. By the sixth day total mortality was 49.8%. Infection of the CPB with *Mr.* resulted in mortality of larvae at 24 (0%), 48 (0%), 72 (10.8%) hours after the treatment. By the ninth day, total mortality was 52%.

Infection of the greater wax moth with Bt resulted in mortality of larvae at 24 (14.6%), 48 (40.2%), 72 (45.6%) hours after the treatment. By the fifth day total mortality was 46.8%. The mortality of greater wax moth was 0%, 8% and 10% at 24, 72 and 120 h respectively following infection with *Bb*. By the fifteen day, total mortality was 52%.

We found that the concentration of DA significantly increased in the haemolymph of CPB larvae during both bacterial and fungal infections at 48 and 72 h post-treatment (Fig. 1). The strong increase in the DA concentration was detected at 48 and 72 h (30-fold and 45-fold, respectively) post-treatment in Bt-infected CPB larvae compared with non-infected individuals (K-W statistic = 36.70, d.f. = 5, $p < 0.01$ for both variants) (Fig. 1a). The concentration of DA significantly increased during *M. robertsii* infection of CPB larvae at 48 and 72 h post-treatment by 4 and 12 times, respectively, compared with the control (K-W statistic = 40.10, d.f. = 5, $p < 0.05$ and $p < 0.001$, respectively) (Fig. 1b). During 72 h of the bacterial infection, the DA concentration was twice as high in the haemolymph of infected wax moth larvae compared with non-infected individuals (K-W statistic = 31.28, d.f. = 5, $p < 0.01$, $p < 0.001$) (Fig. 2a). Moreover, we found the 20-fold increase in the concentration of DA in wax moth larvae individuals infected with *B. bassiana* compared with the control at 120 h after treatment (K-W statistic = 9.80, d.f. = 5, $p < 0.01$) (Fig. 2b).

In this study the half-lethal doses (LD50) were used for insect infection with different pathogens but the dynamics of the infection processes varied between the two insects and depended on the pathogens. For example, bacterial infection leads to the 15% mortality of greater wax moth and 0% mortality of CPB larvae at 24 h after treatment, and DA concentration is elevated only in haemolymph of greater wax moth at this time point. This data indicated that activation of bacterial toxins and infection are developed more slowly in CPB larvae than in greater wax moth and we can detect the delay in elevation of DA concentration. Obtained data show that the concentrations of DA are enhanced from the stage when pathogens penetrate in insect organism through cuticle in case of fungus or midgut in case of bacteria. This stage of fungal and bacterial diseases demonstrated both insect mortality and increase in DA concentration.

The fungal and bacterial infections are stress-induced factors for insects (Butt et al., 2016; Dubovskiy et al., 2016a). Most likely, the detected DA release under both infections is necessary for the development of the general stress reactions related to the behavioural and physiological responses of insects to pathogens. This hypothesis is supported by studies on *Drosophila melanogaster* that demonstrated that the DA is involved in stress reactions, such as heat and cold stress (Rauschenbach et al., 1993; Hirashima et al., 2000). It is known that DA