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Effect of the entomopathogenic fungus *Isaria fumosorosea* on physiological processes in insects

Ph.D. Thesis

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Annotation

The project is focused on a study of the infection elicited by entomopathogenic fungus (EPF) *Isaria fumosorosea* in two model species - the firebug *Pyrrhocoris apterus* and cockroach *Periplaneta americana* - and a role of adipokinetic hormones (AKHs) during that. The AKHs are neuropeptides that belong to the AKH/RPCH family and are known mainly for the mobilisation of energy reserves. The AKH role during the fungal infection was monitored by determination of mortality, gene expression in CNS, level of anti-oxidative stress markers, general metabolism, level of nutrients and activity of digestive enzymes. The *I. fumosorosea* infection elicited severe stress in the infected insects as documented by increase of the AKH expression and AKH levels in CNS of both insect models. The mortality test showed that application of AKH increased the efficacy of EPF application. It is supposed that AKH stimulated higher turnover of fungal toxins by enhanced intensity of metabolism (documented by higher carbon dioxide production) and encouraged the fungus growth by enrichment of haemolymph (a medium for the fungus) by mobilised nutrients. Interestingly, *I.*

fumosorosea affected also digestive processes with substantial inhibition in midgut and partial inhibition in the caeca of male cockroach's digestive enzymes. Another part of this work was to examine changes of the vitellogenin (Vg) level in *P. apterus* after the *I. fumosorosea* treatment. The infection significantly reduced the Vg transcript level (checked by quantitative real-time PCR) in the female fat body and protein level in haemolymph (checked polyacrylamide gel electrophoresis). Interestingly, in the male fat body, Vg transcript level significantly increased after the infection, but EPF did not change protein level of male hemolymph. The obtained results point to an interesting role of Vg in insect defence reactions against EPF and are a good example of a trade-off between the Vg function in reproduction and defence reaction. Results of the project might be useful for better understanding of the mechanism of action of entomopathogenic fungi versus AKH, and could be utilised for practical biocontrol of the insect pests in future.

Declaration [in Czech]

Prohlašuji, že svoji disertační práci jsem vypracoval samostatně pouze s použitím pramenů a literatury uvedených v seznamu citované literatury.

Prohlašuji, že v souladu s § 47b zákona č. 111/1998 Sb. v platném znění souhlasím se zveřejněním své disertační práce, a to v úpravě vzniklé vypuštěním vyznačených částí archivovaných Přírodovědeckou fakultou elektronickou cestou ve veřejně přístupné části databáze STAG provozované Jihočeskou univerzitou v Českých Budějovicích na jejích internetových stránkách, a to se zachováním mého autorského práva k odevzdanému textu této kvalifikační práce. Souhlasím dále s tím, aby toutéž elektronickou cestou byly v souladu s uvedeným ustanovením zákona č. 111/1998 Sb. zveřejněny posudky školitele a oponentů práce i záznam o průběhu a výsledku obhajoby kvalifikační práce. Rovněž souhlasím s porovnáním textu mé kvalifikační práce s databází kvalifikačních prací Theses.cz provozovanou Národním registrem vysokoškolských kvalifikačních prací a systémem na odhalování plagiátů.

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Umesh K Gautam

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DEDICATED TO MY MOTHER

She passed away on 14.04.2018

List of papers and author's contribution

The thesis is based on the following papers (listed chronologically):

I. **Gautam U. K.,** Bohatá A., Shaik H. A., Zemek R., Kodrík D. (2020) Adipokinetic hormone promotes infection with entomopathogenic fungus *Isaria fumosorosea* in the cockroach *Periplaneta americana*. ***Comp Biochem Phys C***. 229: 108677. (IF=2.697)

CONTRIBUTION: Gautam UK was responsible for the experiments and participated in the preparation of the manuscript.

II. Karbusová N., **Gautam U.K.**, Kodrík D. (2019) Effect of natural toxins and adipokinetic hormones on the activity of digestive enzymes in the midgut of the cockroach *Periplaneta americana*. **Arch. *Insect Biochem. Physiol***. 101: e21586. (IF=1.316)

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III. Kodrík D., Ibrahim E., **Gautam U.K.**, Čapková Frydrychová R., Bednářová A., Krištůfek V., Jedlička P. (2019). Changes in vitellogenin expression caused by nematodal and fungal infections in insects. ***J. Exp. Biol***. 222: article no. 202853. (IF=3.017)

CONTRIBUTION: Gautam UK was responsible for some experiments.

Co-author agreement

The corresponding author of the manuscripts included in this thesis hereby confirm that Umesh K GAUATM contributed significantly to these publications, according to the statement above.

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Prof. RNDr. Dalibor Kodrík CSc.

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1. Introduction

1.1. Entomopathogenic fungi and *Isaria fumosorosea*

Entomopathogenic fungi (EPF) are eukaryotic microorganisms, which are heterotrophic, unicellular/multicellular and phylogenetically diverse (Mora et al., 2017). The term entomopathogen was first suggested by German physician Fresenius in 1858: the word “entomopathogen” is derived from two Greek words “entomon” which means “insect” and “pathogen” which is “anything that can produce disease.” Therefore, “entomopathogen” is any microorganism, which develops in insects (Vega et al., 2008). EPF have usually a broad host range. The host range is a set of species that allows survival and reproduction of the EPF. About 1,800 various associations between fungi and different insect species were recorded (Jankevica et al., 2004). Generally, almost all orders of insects can be attacked by EPF, thus, these fungi are commonly used as natural regulators within the biological control of insect pests.

The life cycle of various EPF is very diverse, they can produce sexual or asexual spores, due to this reason they are called as fungi imperfecti. When insects are passing around EPF, the fungi can adhere to the external body surface of insects in the form of conidia and penetrate the exoskeleton (Hajek and St Leger, 1994), nevertheless, conidia are also able to enter the insect haemocoel through the spiracles, mouth and anal openings of insects. These conidia germinate, grow as hyphae and reach the insects' body cavity (haemocoel). Then, the hyphal cells

proliferate in the host as blastospores or hyphal bodies (Vega et al., 2012). The blastospores or hyphal bodies produce toxins that circulate in the haemolymph. After infection, the insect usually dies (sometimes due to fungal toxins or secondary metabolites) and new propagules (conidia) develop on insect cadaver, if environmental conditions are favourable for sporulation (St. Leger et al., 2008). All these steps (adhesion, germination, penetration etc.) are influenced by a range of integrated intrinsic and external factors like various metabolites, enzymes (proteases, chitinases, chitosanases, lipases), temperature and moisture (Ali et al., 2010). Successful infection of EPF is achieved by inhibiting of host regulatory system, and thus by disintegration of homeostasis and by organ malfunction, which causes death of the infected organism. The infection of EPF can be recognised easily by human eyes on the infected cadaver of insects. EPF comes from many orders of fungi kingdom, however, just only a few genera e.g. *Metarhizium*, *Beauveria*, *Isaria*, *Lecanicillium* and *Hirsutella* are used in practice for the insect pest control (Inglis et al., 2000; Faria and Wraight, 2007).

1.1.1. *Isaria fumosorosea*

I. fumosorosea was isolated for the first time from a single larva of the sugar beet weevil *Cleonus punctiventris* in Ukraine (Wize et al., 1904). It was originally known as *Paecilomyces fumosoroseus*, but recently it was renamed and transferred to the genus *Isaria* (Samson et al., 1974; Sung et al., 2007;

Zimmermann et al., 2008). *I. fumosorosea* can be found in soil, dead plants and insect cadaver, where it grows by absorbing nutrients from the decaying matter (Germain et al., 2003). *I. fumosorosea* is a fast-growing fungus producing first white colonies, which change to pink shades on cadaver. The life cycle of *I. fumosorosea* like many other EPF is dimorphic in nature and can exhibit two forms, mycelium (asexual non-motile conidium) and yeast (vegetative fungal), in liquid culture (Deshpande et al., 1999). The yeast-form cells eventually lead to the production of blastospores. The production of conidia starts from conidiogenous cells, these conidia are staying in an environment as in inactive form till the favourable conditions will arrive. The attachment of conidia on the exoskeleton of the host stimulates the germination. Initially, hydrophobicity creates a strong force of interaction between conidia and chitin surface of the host insect. The conidial germination starts with the production of degrading enzymes such as proteases, chitinases, chitosanase, lipase, and then hyphal structures or germ tubes are formed (Ali et al., 2010). These enzymes and development of appressorium (special pathogenic cell type) allow the hyphal structure to breach, pressure, force, penetrate the host insect cuticle, and this process ruptures the outer wall of the host. Finally, the hyphae reach haemocoel of host and produce blastospores. The blastospores undergo budding for rapid propagation and counteracts with the immune system of the hosts (Lord et al., 2002). When available nutrients in the haemolymph of the host have depleted by blastospores then these blastospores start to produce elongating hyphae. These hyphae

produce conidiophores and flask-shaped phialides on the cadaver to complete infection cycle.

As mentioned above, *I. fumosorosea* produces two kinds of spores – blastospores and conidiospores. The blastospores are flask-shaped with a globose to ellipsoidal basal portion, and the aerial conidia are small, compact, hydrophobic, slow germination rate, cylindrical to fusiform (Posada et al., 2004). The blastospores are produced in the haemocoel of infected insects (Jackson et al., 2010), while conidiospores on insect dead bodies (cadaver).

Both blastospores and conidiospores can be produced artificially in culture media for experimental requirements as well as for insect pest control (Jackson et al., 2004). Interestingly, the production of blastospores is faster than that of conidiospores, so this is an advantage of saving time, faster infecting, prevention from growth of bacterial contaminants and having ability to infect the insect. On the other hand, conidiospores are having hard outer wall and their germination rate is compatibly slow due to their hydrophobic nature.

Further, the *I. fumosorosea* spores and its mycelial mass are not the only factors of pathogenicity. The fungus also produces low molecular weight natural compounds, also known as secondary metabolites (SMs). In the last forty years, more than seventy SMs were identified and isolated from *I. fumosorosea* (Weng et al., 2019). The most important are beauvericin, cepharosporolide C, fumosorinone (Inhibitor of PTP1B), trichocarane E, and oxalic acid.

Most of the SMs are considered to be used as natural pesticides. There is a wide range of strains within the species of *I. fumosorosea.* These strains differ from each other in terms of vitality, growth, spore production, germination rates, virulence, and sporulation ability on the surface of the cadaver. They germinate in the temperatures ranging from 19°C to 35°C with 25°C optimum temperature (Cabanillas and Jones, 2009). According to these characteristics, the best strains can be chosen for biological control of pests.

1.1.2. Practical utilisation of *I. fumosorosea*

I. fumosorosea is extensively studied as a biocontrol agent for a number of insect pest species. The target insects include mainly hemipteran such as aphids, *Aphis gossypii* (Jandricic et al., 2014), *Jacobiasca formosana, Stephanitis nashi* (Bugti et al., 2018), *Corythucha ciliata* (Sevim et al., 2013), *Aleurodicus dispersus* (Boopathi et al., 2015), whiteflies, the Asian citrus psyllid *Diaphorina citri* (Zimmermann et al., 2008; Majeed et al., 2017), *Bemisia tabaci* (Boopathi et al., 2015a; Gao et al., 2017). Other insects in this list are lepidopteron insects (mainly moths larvae), such as diamondback moth *Plutella xylostella* (Freed et al., 2012*),* tiger moth *Atteva sciodoxa* (Sajap et al., 2014). *I. fumosorosea* is also effective on the beetles such as rice weevils, *Sitophilus oryzae* (Usanmaz-Bozhuyuk et al., 2018), yellow margined leaf beetles and *Microtheca ochroloma* (Montemayor et al., 2016). Recently, their virulence was shown in order blattodea

the subterranean termites, *Coptotermes curvignathus* and *Coptotermes gestroi* (Jessica et al., 2019), *P.americana* Karbusová et al., 2019; Gautam et al., 2020).

As mentioned above, there are several stains of *I. fumosorosea* available for the insect pest biocontrol. Their germination rate depends mostly on temperature. For example, American strains like Apopka 97, ARSEF 3302, ARSEF 7028 and CNRCB1 usually germinate at high temperature, while European stains such as KTU 42, CCM 8367 and KCh J2 and CBS 107.10 need generally lower temperatures (Zimmermann et al., 2008).

I. fumosorosea strain number CCM 8367 used in our studies (see below Chapter 5. Papers) was isolated from the horse chestnut leaf miner, *Cameraria ohridella* Decka and Dimic (Lepidoptera: *Gracillariidae*) in the Czech Republic and is deposited as a patent culture in the Czech Collection of Microorganisms (CCM) in Brno (Prenerová et al., 2013, Prenerová et al., 2015). The virulence of the strain CCM 8367 has been evaluated in *S. littoralis* (Zemek et al., 2012), spider mite *Tetranychus urticae (*Zemek et al., 2016), Colorado potato beetle *Leptinotarsa decemlineata* (Hussein et al., 2016), cockroach *P. americana* (Karbusová et al., 2019; Gautam et al., 2020) and firebug *P. apterus* (Fig. 1) (Kodrík et al, 2019; Gautam et al unpublished data).

Fig. 1. The effect of *I. fumosorosea* infection on the firebug *P. apterus* adult males after 7 days of the treatment.

From practical reasons the mycelial mass, conidia, blastospores and SMs of *I. fumosorosea* were tested for toxicity on vertebrates. These tests included e.g. the acute dermal toxicity and pathogenicity on rabbits (Brunner-Mendoza et al., 2017), and oral toxicity on frogs and mice (Donovan-Peluso et al., 1980). These studies concluded that no pathological lesions or infection of viscera were caused by *I. fumosorosea*. Thus, *I. fumosorosea* is an effective pathogen that reduces the usage of chemical pesticide and works in the direction which is an environment friendly.

1.2. Stress and insect defence reactions

1.2.1. Stress factors and oxidative stress

The term stress originated in physics to describe pressure and deformation in a system, but it has been adopted into a biological context through the work of Selye et al. (1946), who formulated the “general adaptation syndrome”. Stress is known to disturb the physiological homeostasis of an organism, and to elicit an adaptive response to an ever-changing environment. All organisms including insects are affected by stress in their daily life. Overall, stress negatively affects physiology, growth, development, and changes the homeostasis of the organism with the help of stressors or stress factors. The exogenous or environmental factors responsible for stress in insects, and affecting their development, survival and homeostasis are mentioned in Table 1. These factors induce either acute or chronic stress.

Table 1. Examples of the environmental stressors

1	Chemical factors	pesticides, drugs, metals, smog, abnormal oxygen concentration, etc.
2	Physical factors	radiation, temperature, noise, vibration, humidity, etc.
3	Physiological factors	injury, aging, inflammation, malnutrition, etc.
4	Pathogens	bacteria, viruses, fungi, nematodes, parasitic arthropods, predators, parasitoids, etc.

If these factors are too intensive, this can lead to either abnormal behavioural characteristics or insect paralysis or even insect death (Stenberg and Perkins, 1963). The stress, as a biological phenomenon depends on various parameters, and thus can elicit either specific or nonspecific reaction in insects (Ivanović and Janković-Hladni, 2018). On the other hand, stress can be categorised according to the impact on the target organism: a natural stress which is neither harmful nor helpful, and a bad stress which causes harmful responses (Breazile et al.,1987).

Special example of a stress is oxidative stress (OS); OS is a disturbance in the pro-oxidant–antioxidant balance in favour of the former, leading to potential biomolecular damage caused by an attack of free radicals or generally reactive oxygen species (ROS) upon the constituents of living organisms (Halliwell and Gutteridge, 2007). The overproduction of ROS causes damage to macromolecules like DNA and proteins, and also lipids, and is responsible for cellular malfunction (misfolding of organelles), inhibition of transcription factors, cytoskeleton proteins, molecular chaperons and generally for the degradative machinery (Schonbaum et al., 2000; Dobson et al., 2003). ROS can modulate the gene expression either by down or up regulation (Kim et al., 2002). The primary sources of endogenous ROS are mitochondria, endoplasmic reticulum, and peroxisomes (Moldovan et al., 2004) through a variety of mechanisms including enzymatic reactions and/or autooxidation. The exogenous factors of ROS production are environmental factors

like pesticides, chemicals, UV light and irradiation, etc. (Ahmad et al., 1995). Insects have to respond to the ROS activities; hence, they maintain complex systems of multiple types of antioxidants, such as glutathione, pyruvate, flavonoids, carotenoids, urate, vitamin C, and vitamin E (Valko et al., 2007) as well as enzymes such as catalase, glutathione S-transferase, superoxide dismutase and various peroxidases (Ahmad et al., 1989; Summers et al., 1995; Krishnan et al. 2009).

Obviously, ROS are known for negative (harmful) effects on insects. However, they also act positively on physiological and immunological activities in insects. During the immunological process, microorganisms are eliminated by oxygen-dependent mechanisms, which utilise predominantly H_2O_2 (a type of ROS). Landriscina et al., (2009) showed that ROS are able to modulate the activity of an unconnected set of biochemical reactions, which contribute to migration, survival and cell proliferation. Hydrogen peroxide is involved into the cell signalling mostly as a secondary messenger, and its specific concentration affects the suppression of various genes involved in the T cell immune response, function of mitochondria, iron metabolism, etc. (Morel et al., 1999; Forman et al., 2007).

1.2.2. Insect defence reactions against stress

Insects are exposed to multiple environmental stressors across a variety of habitats, thus, they use many biological response strategies for coping with stress. Whenever homeostatic

is imbalance or threat to homeostasis occurs (Ursin and Eriksen, 2004), responsible sensors alert the brain, which triggers activation of neural and humoral pathways; so, the stress response includes neural, endocrine, cellular and molecular infrastructures. The first and easiest insect anti-stress response is a behavioural one, which means avoiding the stressor by moving away from the threat, which is evidently controlled by sensory and nervous systems (Moberg et al, 1987; Even et al., 2012). Other defence mechanisms are controlled by the endocrine system, where hormones are responsible for initiation of corresponding biochemical reactions. Prime role is played by neuropeptides from special neurosecretory neurones, which can be released directly into the haemolymph, but more frequently they are transported along the axons to corpora cardiaca or other neurohemal organs (Scharrer and Scharrer, 1944). From the neurohemal organs, neuropeptides are released into the haemolymph by exocytosis. The concept of neurosecretion includes not only classical neurosecretory neurons which produce neuropeptides, but also aminergic neurons which produce biogenic amines (Ivanovič and Jankovič-Hladni, 2018).

Neurohormones are key hormones for controlling physiological and behavioural activities of the insect. The negligible amount of neurohormone can modulate physiological processes during the development, reproduction and metabolism, thus, it is obvious that neurohormones are very important for stress reactions (Table 2). For other roles of insect hormones in anti-stress responses see chapter 1.4 insect hormones.

Table 2. Examples of some insect physiological processes mediated by neuropeptides (Holman et al., 1991; Keeley et al., 1991; Gäde et al., 1997b; Resh et al., 2009)

Neurohormones	Action
Homeostasis	
Metabolic peptides (AKH/RPCH family) Adipokinetic hormone (AKH)	Energy mobilisation (see section 1.4.1. for other roles)
Hyperglycemic hormone	Releases carbohydrate from fat body
Hypoglycemic hormone	Enhances carbohydrate uptake
Protein synthesis factors	Enhance fat body protein synthesis
Antidiuretic peptide (ADP)	Suppresses water excretion
Diuretic peptide (DP)	Enhances water excretion
Chloride-transport stimulating hormone	Stimulates Cl^- absorption (rectum)
Ion-transport peptide (ITP)	Stimulates Cl^- absorption (ileum)
Myotropic peptides	
Cardio peptides	Increase heartbeat rate
Kinin family (e.g. leukokinins and myosuppressins)	Regulate gut contraction
Proctolin	Modifies excitation response of some muscles

1.3. Vitellogenin and its role in defence reactions

1.3.1. Vitellogenin as a yolk protein

Vitellogenin (Vg) is oligomeric phosphoglycolipoprotein primarily classified/known as a precursor of yolk proteins - it represents 60 to 90% of soluble egg proteins (Engelmann et al., 1979). Vg was discovered for the first time in the cecropia moth, *Hyalophora cecropia*, (Telfer et al., 1954), and the "vitellogenin" name was given by Pan et al., (1969), after that Vg has been reported in many different groups of insects. The fat bodies are an exclusive site of Vg synthesis in the majority of insects. During vitellogenesis, fat body cells undergo dramatic changes and produce huge amounts of Vg protein in a relatively short time (Keeley et al.,1985; Bellés et al.,1998). After synthesis Vg molecules are secreted into the haemolymph from the fat body, and finally, transferred into developing oocytes through receptor-mediated endocytosis against the concentration gradient; in some species, the Vg is synthesised in follicular cells on ovaria (Zhu et al., 1986, Chapman, 1998). Each insect species possesses one or more vitellogenin polymers that comprise of several polypeptides with the total molecular weight ranging from 150 to 650 kDa. The formation of Vgs is called vitellogenesis – it is a complex process controlled by hormonal and nervous systems. The hormones that are involved in vitellogenesis control, belong to the three classic hormonal families: juvenile hormones, ecdysteroids and neuropeptides (for details see chapter 1.4.). This hormonal system regulating vitellogenesis can vary greatly from group to group at

morphological, physiological, ecological and behavioural levels (Bellés et al., 1998). In the most insect species, JHs play a critical role in vitellogenesis by controlling Vg gene expression; in some species (Dipteran) this is controlled by ecdysteroids. On the other hand, it seems that adipokinetic hormones terminate Vg synthesis (for details see Chapman, 1998).

Mature Vg is transported via haemolymph into the growing oocyte. Upon internalisation by the oocyte, Vg is confined within the clathrin-coated endosomal vesicles (Raikhel et al., 1984). Clathrin is a three-armed protein complex that binds the adaptor complexes, a clathrin lattice self- assembles, in clustering receptors into the clathrin-coated pit (Pucadyil et al., 2016). Once the vesicle is internalised, it is uncoated and clathrin is quickly recycled (Goh et al., 2013). Finally, Vg that gets into the ovarium is called vitellin (Vt), it is a crystalline protein, a source of nutrients during the early development of eggs (Hagedorn and Kunkel, 1979). There is a slight difference between Vg and Vt based on solubility, however, they are identical in molecular weight, and amino acid, lipid and carbohydrate compositions.

Some early works suggested the Vgs were sex-specific and found only in females, but later research has shown varying, but small, amounts of the Vg proteins in males of some species, including representatives of Lepidopteran (*B. mori*, *Antheraea* and *Hyalophora*) *(*Lamy et al., 1984), Diptera (*Drosophila melanogaster)* (King et al., 1965), Blattodean (*P. americana)* (Bell et al., 1972), Orthoptera (*Locusta migratoria*)

(Chinzei et al., 1981), Heteroptera (*P. apterus)* (Němec et al., 1993; Kodrík et al., 2019) and Hymenoptera (*Apis mellifera*) (Trenczek et al., 1986).

1.3.2. Role of Vg in defence reactions

Vg is not only responsible for the egg formation but also plays multiple roles in other aspects of insect biology. Vg is responsible for life-span regulation, caste-differences in social insects (Havukainen et al., 2013; Salmela and Sundstrom, 2017), antimicrobial activity (Kodrík et al., 2019) and also serves as a mediator of transgenerational immunity (Singh et al., 2013; Salmela et al., 2015). The anti-ageing effect of Vg is allowed by its oxidation potential in neutralisation of free radicals in honeybee workers. This function was supported by a number of *in vivo* (Seehuus et al., 2006), and *in vitro* (Havukainen et al., 2013) experiments. Vg molecules also enhance the immune response against bacterial infection by gram-positive and gram-negative bacteria in the silkworm *B. mori* and honeybees *A. mellifera* (Singh et al., 2013). Recently we have found in our study (Kodrík et., al 2019) that Vg proteins play an important role in the defence reactions against nematodal (*Steinernema carpocapsae*) fungal (*I. fumosorosea*) infections in *P. apterus.* The nematodal infection significantly increased expression of *Vg* mRNA in the male fat body and the same tendency was observed on protein level monitored by electrophoresis and immunoblotting. Interestingly, the tendency was opposite in *P.*

apterus females: significant reduction of both *Vg* mRNA and Vg protein expression levels were recorded. We speculate that infection of reproductive females leads to a reduction of Vg to such level that is still effective for the defence, however being off limits for reproduction and therefore, enabling to save energy of the individual to cope with infection. Interestingly, the isolated vitellogenin showed bactericidal activity, when significantly inhibited the growth of *Xenorhabdus* spp. the entomotoxic bacteria isolated from *S. carpocapsae.* On the other hand, the effect of Vg against the fungus *I. fumosorosea* was not so evident. *Isaria* significantly stimulated *Vg* gene expression in males, however, this was not manifested on the protein level. Nevertheless, in females the significant reduction of Vg characteristics after the fungal infection was recorded both on transcriptional as well as on protein levels. The obtained data fit into the previously found characteristics of insect Vg within the insect defence reaction.

1.4. Insect hormones

At the beginning of the invertebrate hormonal research, the endocrinologists expected that this endocrine system is simple, mostly because of smaller invertebrate body size and its simpler organisation in comparison with vertebrates. At this moment with great literature and experimental data that hypothesis was proven to be wrong.

The insect hormonal system consists of several endocrine glands such as prothoracic gland, neurosecretory cells, neurohemal organs (corpora cardiaca (CC), corpora allata (CA)), internal organs (ovaries, testes, ventral ganglia) and glandular cells. These glands are responsible for hormone secretion and hormones control all aspects of insect life such as metabolism, homeostasis, reproduction, development, metamorphosis, moulting or muscle contraction.

The system produces three main groups of insect hormones:

1. Ecdysteroids
2. Juvenile hormones
3. Peptidic neurohormones

1. Ecdysteroids – are steroid hormones released mainly from prothoracic glands, but they are also produced by several other insect organs. Their chemical structure is derived from cholesterol or plant steroids. Ecdysteroids are lipid-soluble, membrane-permeant hormones which penetrate into cells and interact with the intracellular receptors. Ecdysteroids are responsible for moulting, metamorphosis, reproduction, spermatogenesis, metabolism and in certain insect groups also for vitellogenin synthesis (Armold and Regnier, 1975; Adams et al., 1985; Smith et al., 1985).

2. Juvenile hormones (JHs) – JHs are secreted from the CA (Raabe et al., 1989). CA are small, discrete, paired glandular bodies derived from the epithelium and located on either side of the foregut near the brain. In some insects CA fuses to form a single gland. Chemically the JHs are sesquiterpenoids derived

from farnesol. Based on the number of carbon atoms JHs create four types: JH0, JH-I, JH-II and JH-III. JH0 is present only in lepidopteran eggs. JH-I and JH-II are present in Lepidoptera, JH-III is the most common form for all insect species (Beckage and Riddiford, 1983). There are two main functions of JHs in insects: control of metamorphosis and regulation of reproduction. JHs are responsible for maintaining juvenile characteristics in larval body and for inhibition of metamorphosis. In adults they control synthesis of vitellogenin by the fat bodies and its uptake by the developing oocytes (Nijhout, 1994). JHs are necessary to stimulate previtellogenic growth of the primary follicles and to act on the fat body to make it competent to synthesise vitellogenin (Hagedorn, 2013). Additionally, JHs also control other processes like diapause, caste, phase polymorphism, pheromone production and protein synthesis, etc.

3. Peptidic neurohormones – the most numerous groups of insect hormones. Neurohormones are secreted by specialised neurones in the brain, CC and ventral nerve cord. They may act directly on effector organs or on other endocrine glands to control their hormonal production. Peptide hormones are the master regulators of all insect physiological processes, including development, homeostasis, metabolism and reproduction, as well as the secretion of the JHs and ecdysteroids. Several hundred neuropeptides have been recognised so far, many existing in multiple forms encoded by the same gene but resulting from gene duplication or mutation (Gullan and Cranston, 2014). The neurohormones classification is not uniform but can be

categorised according to their function as (a) metabolic and homeostatic controlling hormones, (b) reproduction controlling hormones, (c) development, growth and metamorphosis controlling hormones, (d) chromatotropins controlling hormones and (e) muscle contraction modulating hormones.

A very important group of neurohormones is adipokinetic hormones (see below).

1.4.1. Adipokinetic hormones

The adipokinetic peptides, members of the adipokinetic hormone/red pigment concentrating hormone (AKH/RPCH) arthropod peptide family, are a typical example of neuropeptides with complex functions. AKHs are hydrophobic compounds and are stable at high temperature. The AKH peptides are synthesized and stored in the CC or in the corresponding CC cells of dipteran ring glands from where they are released into the haemolymph when necessary (Kim and Rulifson, 2004).

The first AKH structure was published by Stone et al., (1976) at the Locmi-AKH-I – one of the three AKHs in *Locusta migratoria*. Generally, AKH peptides contain from 8 to 10 amino acid residues (Gäde et al., 1997a). All these peptides are N-terminally blocked by a pyroglutamate (pGlu) residue and C terminally amidated (Van der Horst et al., 2003). Köllisch et al. (2000) isolated and sequenced an unusual AKH from the butterfly *Vanessa cardui* (Vanca-AKH-I), it has got 11 amino acids with a non-amidated C-terminus. More than 60 isoforms of AKHs have

been identified from different insect orders so far (Kaufmann et al., 2009; Gäde et al., 2015) and the list is growing continuously. The organisation of AKH molecules show some general features: the amino acids tryptophan and glycine are at position 8 and 9 (when present); in addition to tryptophan, the molecule contains at least one more aromatic amino acid, most commonly phenylalanine at position 4.

It has been shown in some studies that neurotransmitter octopamine somehow relates to the AKH release from CC because the octopamine containing neurons make synaptic contact with the adipokinetic cells (Orchard et al., 1993). Further, Diederen et al., (2002) showed that the haemolymph titre of octopamine is known to increase at the onset of flight, which supports the findings of Passier et al., (1995), who suggested that octopamine stimulates adipokinetic cells to release the AKH. The same role was also suggested for other amines as dopamine, serotonin and tyramine (Passier et al., 1995).

As mentioned above, the AKH is primarily synthesised in the CC, but a small amount can be found also in the brain. The presence of AKH in insect brain has been confirmed in several species by immunohistochemistry (Schooneveld et al., 1985; Bray et al., 1993; Kodrík et al., 2003;). Nevertheless, Patel et al., (2014) recently questioned these results by presenting data obtained in *Rhodnius prolixus,* claiming that immunological evidence of AKH presence in insect brain is due to cross-reactivity of the AKH antibody with corazonin or adipokinetic hormone/corazonin-related peptide (Patel et al., 2014). However,

this contradiction was solved by studies on *P. apterus* (Kodrík et al., 2015a, b), where it has been proven that AKHs are synthesised both in the CC and also in brain. There are more insects at present showing AKH synthesis in the brain: e.g. *Carausius morosus* and *Sarcophaga bullata* (Clottens et al., 1989) and *Aedes aegypti* (Kaufmann et al., 2009). The performances of the AKH family representatives are quite diverse and certainly broader than what their name implies (Gäde et al., 1997; Kodrík et al., 2008). The primary and classical function is a mobilisation of nutrient reserves from the fat body to provide energy substrates for various energy-demanding processes (Goldsworthy et al., 1983). However, the AKH family is involved in many more activities listed in Table 3.

The mode of AKH action on subcellular level has been intensively studied. AKHs as peptidic hormones are not able to penetrate freely the cell membrane, and thus their message is transduced via specific membrane receptors (Park et al., 2002; Staubli et al., 2002; Kaufmann and Brown, 2006). The outcome of AKH signal transduction through this receptor is well documented for the metabolism-stimulating processes (Gäde and Auerswald, 2003). The process involves a classical intracellular cascade typical for peptide hormone signalling via the adenylate cyclase or phospholipase C pathways, both involving the presence of extracellular and/or intracellular Ca2+ in presence of inositol triphosphate and diacylglycerol (Gäde and Auerswald, 2003). AKHs are responsible for hyperlipaemic effect: here the second messenger cyclic AMP stimulates lipase activity via activation of

Table 3. Main activities of adipokinetic hormones in insect body

Role of AKHs	**Source**
Lipid mobilisation	-
Carbohydrate mobilisation	-
Proline synthesis	-
Activation of adenylate cyclase and lipase	(Spencer and Candy, 1976)
Activation of phospholipase C	(Vroemen et al., 1997)
Activation of glycogen phosphorylase	(Marrewijk et al., 1980)
Stimulation of mitochondrial cytoheme biosynthesis	(Keeley et al., 1991)
Increase of the lipid-carrying capacity of lipoprotein carriers	(Kanost et al., 1990)
Activation of antioxidant mechanisms	(Kodrík et al., 2015a, 2007; Večeřa et al., 2007)
Inhibition of RNA synthesis	(Kodrík and Goldsworthy, 1995)
Inhibition of protein synthesis	(Carlisle and Loughton, 1979)
Inhibition of lipid synthesis	(Gokuldas et al., 1988)
Stimulation of heartbeat	(Scarborough et al., 1984)
Increasing of muscle tonus	(O'Shea et al., 1984)
Stimulation of general locomotion	(Socha et al., 1999)
Enhancement of immune responses	(Goldsworthy et al., 2002)
Inhibition of egg maturation	(Lorenz et al., 2003)
Regulation of starvation-induced foraging behaviour	(Lee and Park, 2004)
Stimulation of neuronal signaling	(Milde et al., 1995)
Enhancement of food intake and digestive processes in insect gut	(Kodrík et al., 2012; Bil et al., 2014; Vinokurov et al., 2014; Bodláková et al., 2017, 2018; Karbusová et al., 2019)

a protein kinase A (Arrese et al., 1996). The active lipase breaks down triacylglycerols to diacyglycerols that represent the insect transport form of lipids in the haemolymph (Gäde and Auerswald, 2002b). The whole process is dependent on entry of extracellular and intracellular Ca2+ release into the cytosol (Vroemen et al., 1997).

Interestingly, AKH peptides also cause hyperprolinaemia (Gäde and Auerswald, 2002a) that can be considered as a side pathway of the hyperlipaemic effect. Proline is used as a metabolic substrate for flight in some fly and various beetle species. The information of the signalling pathway for activation of proline synthesis has been gathered mostly from investigations on the fruit beetle *Pachnoda sinuata* (Auerswald and Gäde, 2001).

AKHs also mobilise trehalose, trehalose is a transport form of carbohydrates, whenever energy needed it is a first metabolite that is hydrolysed. Which is transported to working muscle cells. In muscles, trehalose is converted back into glucose and utilised as a source of energy (Arrese et al., 1999; Gäde & Auerswald, 2003).

However, the mechanism by which AKHs exert their other functions is mostly unknown; just recently it has been found that both mentioned signalling pathways (adenylate cyclase and phospholipase C) are probably simultaneously utilised in the anti-oxidative stress reaction in the firebug *P. apterus* (Bednářová et al., 2013).

Several AKH functions are described in detail in the following text because those functions were investigated within my Ph.D. study and are involved in the corresponding papers of this thesis.

1.4.1.1. AKH role in digestive processes

Insect digestion is a set of complicated processes controlled by nervous and endocrine systems. Several hormones are involved into the stimulation (for details see Audsley and Weaver, 2009), and an important role is played by AKHs. The role of AKH in the digestive system was demonstrated in *P. apterus*, where this hormone increased the activity of some digestive enzymes and the food consumption as well (Kodrík et al., 2012; Vinokurov et al., 2014). The injection of AKH increased the level of glucosidase and peptidase enzymes in midgut of *P. apterus* significantly but lipase activity was not changed (Kodrík et al., 2012). Furthermore, AKH digestive activity was tested in *P. americana* (Bodláková et al. 2017, 2018; Karbusová et al., 2019) Bodláková et al. (2017) showed that the amylase activity increased in gut and ceca after the application of AKH under both *in vivo* as well as *in vitro* conditions. Further, a study in *Sarcophaga crassipalpis* (Bil et al., 2014) confirmed role of AKH in digestion. Vinokurov et al., (2014) showed that the *P. apterus* salivary glands did not participate in digestive reactions but AKH modulates the activity of salivary glands and polygalacturonase enzymes. The mechanism of AKH action is not known here but probably cAMP

participates in the signalling pathway. Interestingly, AKH was able to reverse the inhibiting effect of some natural toxins on insect digestion, however, this was not recorded when the blastospores of *I. fumosorosea* were tested. They decreased the activity of digestive enzymes (amylase, lipase and protease) in the midgut of *P. americana (*Karbusová et. al. 2019), but co-application with AKH did not change the inhibition. These findings suggest that AKHs activity on digestive system is species and probably also pathogen/toxin specific. Further experiments are needed for better understanding this phenomenon.

1.4.1.2. AKH role in oxidative stress

Insects use various antioxidant strategies to protect themselves from OS (for details see also Chapter 1.2.2). It has been suggested recently that an important role is also played by AKHs. This phenomenon was firstly described in the paper of Kodrík et al., (2007) and later on confirmed by a number of other publications (for review see Krishnan and Kodrík, 2012; Kodrík et al., 2015a, b). Generally, the AKH level increased significantly in CNS/haemolymph of insects under OS conditions *in vitro* and *in vivo (*Bednářová et al., 2013). Further, AKHs modulated activity of anti-OS enzymes like superoxide dismutase (SOD), glutathione S transferase (GST) and catalase (CAT) as well as a level of non-enzymatic OS markers like glutathione (GSH), protein carbonylation, lipid peroxidation or total antioxidant capacity. Accordingly, the oxidative stressor paraquat

(1,1'-dimethyl-4,4'-bipyridylium dichloride hydrate) increased the AKH level in the CNS of *D. melanogaster* about twice 24h post-injection (Zemanová et al., 2016) and in *Leptinotarsa decemlineata* CNS about 2 times 4 h post-injection (Kodrík et al., 2007). Corresponding increase in the level of AKH was also recorded in haemolymph of the mentioned species (Večeřa et al., 2007; Kodrík et al., 2007).

These findings were also confirmed with the injection of other stressors (as hydrogen peroxide, several insecticides with OS effect or tannic acid) into *P. apteru*s (Velki et al., 2011), *S. littoralis* (Večeřa et al., 2012) or *Tribolium castaneum* (Plavšin et al., 2015). Interestingly, *I. fumosorosea* treatment probably elicits status of OS in the infected insect body (Gautam et al., 2020): we have found that this EPF decreased the catalase activity in the cockroach *P. americana* body and that co-application with AKH increased it back to or even above the control level.

1.4.1.3. AKH stimulation of insecticide and pathogen efficacy

Natural toxins and pathogens as well as 'artificial' insecticides elicit severe stress in insect organisms. Thus, it is not surprising that AKHs are involved also in this matter. Originally, it was expected that AKH could help to reduce the harmful effect of toxins and pathogens on insect body. However, this prerequisite turned out to be untrue. It has been found that EPF caused a certain level of the mortality in insect model. However, the co-application of AKH with EPF significantly increased this

level (Gautam et al., 2020). The real mechanism is not known, but we can hypothesize that: (1) the intensification of metabolism by AKH might increase turnover of toxins from the pathogens; the metabolism increase was experimentally confirmed by elevation of CO_2 production, (2) mobilisation of nutrients in haemolymph by AKH might provide better substrate for more intensive pathogens growth. and (3) maybe also inhibition of synthesis of anti-microbial peptides could play a role. The first indication of this phenomenon was recorded by the Goldsworthy group. The application of laminarin activated the prophenoloxidase cascade in *L. migratoria* and the effect was strengthened after co-application of laminarin with Locmi-AKH (Goldsworthy et al., 2002). Furthermore, application of lipopolysaccharide from *Escherichia coli* induced the formation of nodules in the same model species. As expected, AKH co-application with LPS increased the number of nodules being formed and activated also the prophenoloxidase cascade (Goldsworthy et al., 2003). And finally, Mullen and Goldsworthy (2006) found that conidia of *M. anisopliae* showed higher mortality when injected into locusts with AKH.

Recently, a similar effect was also recorded in our laboratory when other pathogens were tested with AKHs. We found that the entomopathogenic nematode *S. carpocapsae* (Ibrahim et al., 2017), and entomopathogenic fungus *I. fumosorosea* (Gautam et al. 2020) showed significantly higher mortality in insect model species when applied with AKHs. These findings were confirmed by the corresponding results: increase of

production of CO_2 and increase of mobilised nutrients in haemolymph. As expected, both pathogens also increased the level of AKH in the experimental insects. AKHs also increased the efficacy of insecticides (Kodrík and Socha, 2005; Kodrík et al., 2010; Velki et al., 2011; Plavšin et al., 2015). The reasons are probably similar as were explained for pathogens.

The above-mentioned results indicate that the idea of stimulation of insecticide and pathogen efficacy by insect hormones might be interesting in insect pest control in the future. Of course, we are far from practical utilisation, but a complete understanding of the mechanism of AKH action, and future experiments could suggest further directions of the research.

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3. Objectives – The aim of the study

The main aim of the study was to investigate a role of the adipokinetic hormone (AKH) and vitellogenin in infections elicited by entomopathogenic fungi *I. fumosorosea*. For those various markers were monitored: (1) mortality of experimental insects after application of the EPF alone and co-application of the EPF with AKH; (2) levels of basic nutrients (carbohydrates, lipids, proteins); (3) activity of digestive enzymes (proteases, lipases, amylases); (4) level of CO_2 production as a marker of metabolism intensity; (5) changes of anti-oxidative stress markers; (6) AKH gene expression, and AKH level in CNS and haemolymph; and (7) expression of Vg gene and level of Vg proteins in haemolymph and Vg role in defence mechanisms of *I. fumosorosea* treated insects.

4.1. Results

The results of this thesis are included in three published papers.

Paper I

Gautam U.K., Bohatá A., Shaik HA., Zemek R., Kodrík D. (2020) Adipokinetic hormone promotes infection with entomopathogenic fungus *Isaria fumosorosea* in the cockroach *Periplaneta americana*. ***Comp Biochem Phys C***. 229: 108677.

The AKHs are known to be involved in insect immunity, thus their role in the cockroach *P. americana* infected with the entomopathogenic fungus *I. fumosorosea* was examined in this study. The application of *I. fumosorosea* resulted in a significant increase in both Akh gene expression and AKH peptide levels. Further, co-application of *I. fumosorosea* with Peram-CAH-II significantly enhanced cockroach mortality compared with the application of *I. fumosorosea* alone. The mechanism of AKH action could involve metabolic stimulation, which was indicated by a significant increase in carbon dioxide production; this effect can increase the turnover and thus efficacy of toxins produced by *I. fumosorosea* in the cockroach's body. *I. fumosorosea* treatment resulted in a significant decrease in haemolymph nutrients (carbohydrates and lipids), but co-application with Peram-CAH-II restored control level of lipids or even further increased the level of carbohydrates. Such nutritional abundance could enhance the growth and development of *I. fumosorosea*. Further, both *I.*

fumosorosea and Peram-CAH-II probably affected oxidative stress: *I. fumosorosea* alone curbed the activity of catalase in the cockroach's gut, but co-application with Peram-CAH-II stimulated it. Interestingly, the hormone alone had no effect on catalase activity. Taken together, the results of the present study demonstrate the interactions between the fungus and AKH activity; understanding this relationship could provide insight into AKH action and may have practical implications for insect pest control in the future.

Paper II

Karbusová N., **Gautam U.K.**, Kodrík D. (2019) Effect of natural toxins and adipokinetic hormones on the activity of digestive enzymes in the midgut of the cockroach *Periplaneta americana*. **Arch. *Insect Biochem. Physiol***. 101: e21586.

This study examined the effect of two natural toxins (a venom from the parasitic wasp *Habrobracon hebetor* and destruxin A from the entomopathogenic fungus *M. anisopliae*), and one pathogen (the entomopathogenic fungus *I. fumosorosea*) on the activity of basic digestive enzymes in the midgut of the cockroach *P. americana*. Simultaneously, the role of AKH in the digestive processes was evaluated. The results showed that all tested toxins/pathogens elicited stress responses when applied into the cockroach body, as documented by an increase of AKH level in the central nervous system. The venom from *H. hebetor* showed no effect on digestive enzyme activities in the ceca and

midgut *in vitro*. In addition, infection by *I. fumosorosea* caused a decrease in activity of all enzymes in the midgut and a variable decrease in activity in the ceca; application of AKHs did not reverse the inhibition. Destruxin A inhibited the activity of all enzymes in the midgut but none in the ceca *in vitro*; application of AKHs did reverse this inhibition, and no differences between both cockroach AKHs were found. Overall, the results demonstrated the variable effect of the tested toxins/pathogens on the digestive processes of cockroaches as well as the variable ability of AKH to counteract these effects.

Paper III

Kodrík, D., Ibrahim, E., **Gautam, U. K.**, Čapková Frydrychová, R., Bednářová A., Krištůfek V., Jedlička P. (2019) Changes in vitellogenin expression caused by nematodal and fungal infections in insects. ***J. Exp. Biol***. 222: article no. 202853.

This study examined the expression and role of vitellogenin (Vg) in the body of the firebug *Pyrrhocoris apterus* (Heteroptera, Insecta) during infection elicited by two entomopathogenic organisms, the nematode *Steinernema carpocapsae* and the fungus *Isaria fumosorosea*. Infection by *S. carpocapsae* significantly upregulated *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 opposite tendency was noted. 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 a level that is still sufficient for defense, but is 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 at 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 probably 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.

4.2. Conclusions

The main results are concluded as follows:

1. Application of the entomopathogenic fungus *Isaria fumosorosea* increases natural level of AKHs in the cockroach *Periplaneta americana.*
2. External AKH is able to increase the efficacy of *I. fumosorosea* in this cockroach.
3. The increase of efficacy probably involves: (i) stimulation of metabolism, which can increase turnover of fungal toxins, (ii) enrichment of haemolymph - a medium for the fungus growing – by nutrients, and (iii) maybe also inhibition of synthesis of anti-microbial peptides.
4. AKHs generally stimulate insect gut functions but are not able to eliminate the negative effect of *I. fumosorosea* on the cockroach gut. This phenomenon is unclear, probably species and pathogen specific.
5. Vitellogenin protein protects the firebug *Pyrrhocoris apterus* against the entomopathogens – fungus *I. fumosorosea* and nematode *Steinernema carpocapsae* including its entomotoxic bacteria.

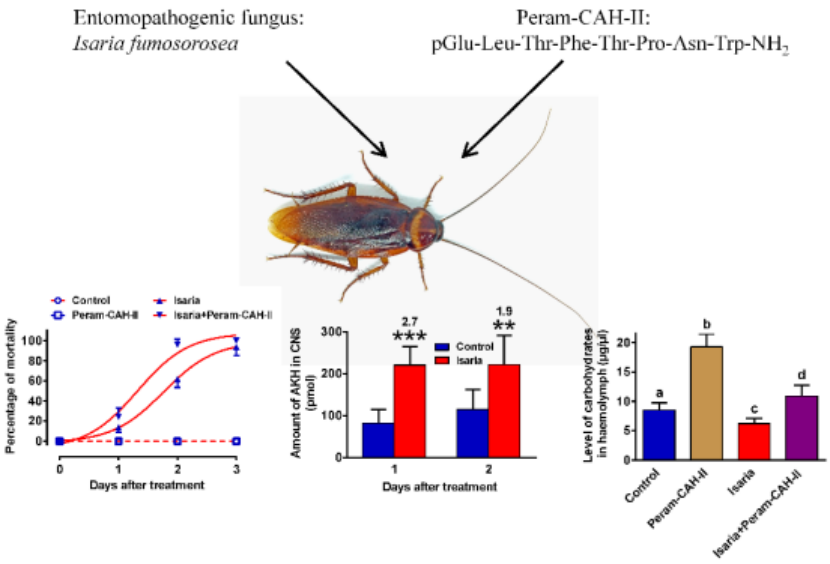
5. Papers


Archives of
INSECT BIOCHEMISTRY
AND PHYSIOLOGY
Journal of
Experimental Biology
The Company of
Biologists
COMPARATIVE BIOCHEMISTRY AND PHYSIOLOGY
CBP
Toxicology
&
Pharmacology

5.1. Paper I

Gautam U.K, Bohatá A., Shaik H.A., Zemek R., Kodrík, D. (2020) Adipokinetic hormone promotes infection with entomopathogenic fungus *Isaria fumosorosea* in the cockroach *Periplaneta americana*. ***Comp Biochem Phys C***. 229: 108677.

Graphical abstract:



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Adipokinetic hormone promotes infection with entomopathogenic fungus *Isaria fumosorosea* in the cockroach *Periplaneta americana*

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ABSTRACT

The adipokinetic hormones (AKHs) are known to be involved in insect immunity, thus their role in the cockroach *Periplaneta americana* infected with the entomopathogenic fungus *Isaria fumosorosea* was examined in this study. The application of *I. fumosorosea* resulted in a significant increase in both *Akh* gene expression and AKH peptide levels. Further, co-application of *I. fumosorosea* with Peram-CAH-II significantly enhanced cockroach mortality compared with the application of *I. fumosorosea* alone. The mechanism of AKH action could involve metabolic stimulation, which was indicated by a significant increase in carbon dioxide production; this effect can increase the turnover and thus efficacy of toxins produced by *I. fumosorosea* in the cockroach's body. *I. fumosorosea* treatment resulted in a significant decrease in haemolymph nutrients (carbohydrates and lipids), but co-application with Peram-CAH-II restored control level of lipids or even further increased the level of carbohydrates. Such nutritional abundance could enhance the growth and development of *I. fumosorosea*. Further, both *I. fumosorosea* and Peram-CAH-II probably affected oxidative stress: *I. fumosorosea* alone curbed the activity of catalase in the cockroach's gut, but co-application with Peram-CAH-II stimulated it. Interestingly, the hormone alone had no effect on catalase activity. Taken together, the results of the present study demonstrate the interactions between the fungus and AKH activity; understanding this relationship could provide insight into AKH action and may have practical implications for insect pest control in the future.

1. Introduction

Organismal defence response against pathogens is primarily considered a reaction of the nervous and endocrine systems that allows an organism to eliminate or at least to reduce the perturbations of bodily homeostasis induced by the pathogens (Storey, 2004). During the response, feeding and reproduction are curtailed, heart and gas exchange rates increase, metabolic processes release stored energy, and oxygen and nutrients are directed to the sites in the body that are most affected. In mammals, the defence response pathways are controlled by the hypothalamic–pituitary–adrenal axis with the help of a suite of corresponding hormones. In insects, the biochemical and physiological defence reactions under unfavourable conditions are controlled predominantly by adipokinetic hormones (AKHs) and some other neuropeptides. The molecular mechanisms of the defence responses of AKH on the cellular and subcellular level in insects, however, remain insufficiently understood.

The principal function of AKH peptides lies in the control of energy metabolism, which is documented by dozens of studies and reviews (e.g. Gäde et al., 1997; Gäde and Goldsworthy, 2003); accordingly, AKHs behave as typical stress hormones by stimulating catabolic reactions (mobilising lipids, carbohydrates, and proline) and increasing energy availability. Nevertheless, AKHs, as pleiotropic hormones, exhibit substantially more activities, which boosts their main roles in energy metabolism (e.g. Nässel, 2002; Kodrík, 2008; Kodrík et al., 2015).

It is generally accepted that AKHs play a role in insect defence reactions against various pathogens, insecticides, and natural toxins. The role of AKH in insect immunity was first documented by Goldsworthy et al. (2002a) in *Locusta migratoria*. The authors reported that injection of laminarin (an alga-derived immunogen fairly typical of the β-1,3-glucans present in fungal cell walls) activated the prophenoloxidase cascade in the haemolymph, and this activation was enhanced when locust AKH (Locmi-AKH-I) was co-injected with laminarin. Further, the

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injection of bacterial lipopolysaccharide from *Escherichia coli* stimulated the formation of nodules but did not increase phenoloxidase activity in the haemolymph. Co-injection of Locmi-AKH-I and lipopolysaccharide resulted in the formation of a greater number of nodules and activated the prophenoloxidase cascade (Goldsworthy et al., 2003a, 2003b). Recently, several other detrimental factors (insecticides, toxins, and pathogens) were found to significantly increase the level of AKHs in the insect body, also suggesting hormonal involvement in the defence reactions (Kodrík et al., 2010; Velki et al., 2011; Plavšin et al., 2015; Ibrahim et al., 2017; Shaik et al., 2017). On the other hand, co-application of those factors with AKH significantly increased mortality in treated insects. The exact mechanism underlying this phenomenon is not known, but it is speculated that the stimulation of metabolism by AKH can intensify the toxin action via an accelerated rate of exchange of metabolites accompanied by their faster penetration into cells. In the case of living pathogens, an increase in nutrient levels in the haemolymph by AKH may play a decisive role.

The majority of insects possess only one AKH, but in several species, two or even three AKHs exist. In *Periplaneta americana* (L., 1758), two AKHs have been described: Peram-CAH-I (*Periplaneta americana* cardioaccelerating hormone I: pGlu-Val-Asn-Phe-Ser-Pro-Asn-Trp-NH_2) and Peram-CAH-II (pGlu-Leu-Thr-Phe-Thr-Pro-Asn-Trp-NH_2) (Scarborough et al., 1984); these molecules differ in three amino acids. The practical implications of this phenomenon are not known, despite some indications of partial functional specialisation of AKHs in the insect body (e.g. Kodrík and Goldsworthy, 1995; Candy, 2002; Kodrík and Socha, 2005).

Isaria fumosorosea Wize (Wize, 1904) (Hypocreales: Cordycipitaceae) is an entomopathogenic fungus which has a worldwide distribution: its natural occurrence in soil samples from many countries has been reported (Zimmermann, 2008; Sevim et al., 2010; Medo and Cagáň, 2011). It is a species with a relatively wide host range across several insect orders and it therefore has received significant attention as a possible biological control agent for several economically important insect pests of agricultural crops. Several commercial mycoinsecticides based on various strains of this fungus have been developed in America, Asia and Europe (Zimmermann, 2008). *I. fumosorosea* initiates attack on the host by penetration of the cuticle. To do so, various enzymes such as proteases, lipases, chitinases, and chitosanases are employed (Hajek and Leger, 1994; Ali et al., 2010). In the host's body, the fungus dissolves the tissue and organs with these enzymes to obtain nutrients for its growth and development. *I. fumosorosea* also produces the toxin depsipeptide—known as beauvericin—that facilitates infection in the host body by killing the infected cells (Luangsa-Ard et al., 2009). It is likely that *I. fumosorosea* interacts with the insect endocrine system. Recently, we showed that *I. fumosorosea* infection elicited substantial inhibition of the digestive processes in the cockroach midgut, with partial inhibition in the caeca (Karbusová et al., 2019). External application of AKHs did not reverse this inhibition, although the fungus elevated the AKH level in the brain of *P. americana* 1 day after treatment. Despite this, the same inhibitory effect of destruxin A, a toxin from the entomopathogenic fungus *Metarhizium anisopliae*, was abolished by AKHs (Karbusová et al., 2019). Interestingly, *M. anisopliae* also affected the defence system of the locust L. *migratoria*, and the injection of Locmi-AKH-I further enhanced the efficacy of the fungus conidia, resulting in increased locust mortality (Goldsworthy et al., 2005). The higher turnover of toxins and increased nutrient content in the haemolymph are possibly involved in this effect.

The main goals of this study were to examine the putative role of AKH in infection by the fungus *I. fumosorosea* and to characterise the physiological processes affected by the infection. We focused on the verification of the role of the fungal infection, which is potentially able to elicit similar AKH reactions as recently recorded for other detrimental factors, and thus contribute to the expansion of the list of known AKH functions.

2. Materials and methods

2.1. Insects and their rearing

A mass culture of the American cockroach *Periplaneta americana* was used in the present study. The cockroaches were kept in 60 l glass fish tanks under 12 h L: 12 h D circadian conditions at constant temperature of 30 ± 1 °C, and were supplied with food (apples, carrots, stale bread, oat flakes) and water ad libitum. To avoid possible complications with the female ovarian cycle, only active males (with hardened cuticle and without external damages) of undefined age were used for the experiments. Pilot experiments showed no significant difference in sensitivity to *Isaria* infection between young and old cockroach males (Fig. S1).

2.2. Entomopathogenic fungus

For this study the strain CCM 8367 of *I. fumosorosea* was used. The strain originated from infected pupae of the horse chestnut leaf miner, *Cameraria ohridella* was collected in the Czech Republic (Zemek et al., 2007) and is deposited as a patent culture in the Czech Collection of Microorganisms (CCM) in Brno (Prenerová et al., 2013, 2015). The fungus was cultivated on potato dextrose agar medium in the dark at 25 ± 1 °C for 14 days. Basic conidial suspension was prepared by soaking of full sporulated culture with a sterile solution of 0.05% (v/v) Tween 80® (Sigma-Aldrich), and conidia were scraped using an inoculative loop into Erlenmeyer flask. The concentration of conidia was quantified using Improved Neubauer counting chamber (Marienfeld, Germany) and its suspension consequently adjusted to the concentration 5.0×10^6 conidia per ml. For blastospore production, the suspension was diluted 20-times in sterile potato dextrose and incubated using an orbital shaker with speed of 200 rpm at 25 °C and constant light for 4 days. Thereafter the blastospore biomass was transferred into the sterile plastic tubes and washed twice with sterile distilled water. Blastospore density was measured using the counting chamber and adjusted to the stock concentration 5.0×10^6 conidia per 1 ml. The germination of fresh conidia and blastospores was evaluated using a standard germination test (Skalický et al., 2014): ten drops from each conidia or blastospore suspension were applied using a 1 µl inoculation loop on the surface of 2% water agar, which was poured in a thin layer onto the surface of a sterile slide. After the drops had dried, the slides were moved into a wet chamber and incubated 25 ± 1 °C. The germination capacity of the conidia and blastospores was evaluated within 24 h using the Olympus light microscope. The results were expressed in percentage of germination.

As the blastospores proved to germinate significantly faster than conidia (Fig. 1), we used *I. fumosorosea* blastospores for our experiments.

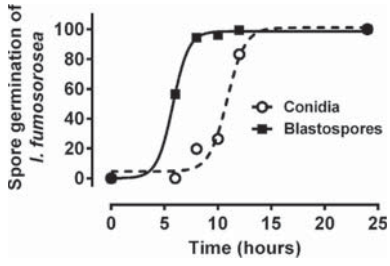

Fig. 1. Percentage of germination of *I. fumosorosea* conidia and blastospores. For details see Materials and Methods section. Repeated measures ANOVA with the Tukey's post-test revealed significant difference between the groups at 0.1% level; $n = 3$ groups with 100 conidia or blastospores in each.

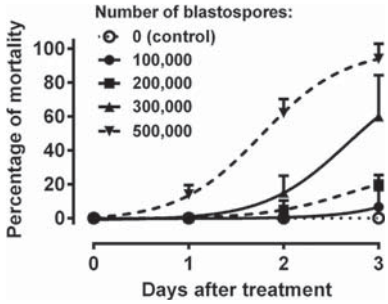

Fig. 2. The effect of increasing doses of *I. fumosorosea* blastospores on mortality of *P. americana* adult males 1, 2 and 3 days after the treatment. Repeated measures ANOVA with the Tukey's post-test revealed significant differences among all treatments with the exception of control vs. 100,000 blastospores, at minimally 5% level; $n = 4$–5 groups with 20 individuals in each.

2.3. Cockroach treatments and dissection

In the general experiments, cockroaches were injected by a dose of 500,000 *I. fumosorosea* blastospores per individual in 5 µl Ringer saline through intersegmental membrane between rear abdominal segments into the abdominal haemocoel; this dose was determined by initial experiment (see Fig. 2). Controls were injected with Ringer saline only. All treated cockroaches were kept under the same conditions as mentioned for the mass cockroach culture in chapter 2.1. The experiments were terminated 1, 2 or 3 days later by cooling down the cockroaches on ice and their dissection (see below).

In some experiments the effect of Peram-CAH-II (Scarborough et al., 1984) was monitored. This peptide was commercially synthesized in the Polypeptide Laboratories, Praha, and a dose of 40 pmol (Bodláková et al., 2017) was injected (in 2 µl) into the cockroach bodies. To monitor the effect of *I. fumosorosea* and Peram-CAH-II, a usual design of the experiments was: (a) control, (b) *I. fumosorosea* alone, (c) Peram-CAH-II alone, and (d) *I. fumosorosea* + Peram-CAH-II together.

To determine mortality, four or five groups (each consisting of 20 cockroaches) for each experimental treatment were inspected 1, 2 and 3 days post treatment.

For dissection the cockroaches were anesthetized on ice, then the haemolymph was collected from cut antenna or leg, and CNS (brain + corpora cardiaca + corpora allata) and midgut were dissected with fine forceps. The organs were processed as mentioned below.

2.4. Expression of Peram-Cah-II gene

- *The CNS preparation*. The CNS from *P. americana* males were dissected under a stereomicroscope in sterile, ice-cold RNAase-free Ringer solution in sterilized glass Petri dishes placed on crushed ice. CNS of four *P. americana* individuals were pooled as one replicate. Immediately after dissection the CNS 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). 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 Peram-Cah-II gene expressions.* One q-RT-PCR reaction mixture contained 7 µl SYBR® Premix Ex Taq™II (TaKaRa), 3 µl of 10× diluted cDNA template, 500 nM forward and reverse primers, and water in total volume of 14 µl. The gene specific primers, forward (5′- GAG GTC GGG ACT CCA AGA TG −3′) and reverse (5′- AAC TTC CCA CAC TCC ACC AG −3′) to amplify *Peram-Cah-II* were used, respectively. The sequence encoding actin was amplified with forward (5′- CAC GGT ATC GTG ACC AAC TG −3′) and reverse (5′- GAA AGC ACA GCC TGG ATA GC -3′) primers and used as reference gene. The reaction was performed on the Light Cycler (Bio-Rad, USA) and the program was as follows: initial denaturation step at 95 °C for 3 min, followed by 40 cycles of denaturation at 94 °C for 15 s, annealing at 60 °C for 30 s and elongation at 72 °C for 30 s. A final melt-curve step was included post-PCR (ramping from 65 °C to 95 °C by 0.5 °C every 5 s) to confirm the absence of any non-specific amplification. The efficiency of each primer pair was assessed by constructing a standard curve through four serial dilutions. Each q-RT-PCR experiment consisted of six independent biological replicates with three technical replicates for each parallel group. The reaction efficiency and Ct values were analysed using Bio-Rad CFX Manager software. Relative gene expression was determined using the method described by Pfaffl (2001).

2.5. Quantification of Peram-CAH-I and -II by ELISA

A competitive ELISA was used for quantification of total AKH content (Peram-CAH-I and -II together) in *P. americana* CNS. Our protocol published earlier included rabbit antibodies that were originally raised commercially against Cys^1-Pyrap-AKH (Sigma Genosys, Cambridge, UK), however, the antibody recognised well both the Peram-CAH-I and –II (Goldsworthy et al., 2002b; Karbusová et al., 2019). In the experiments one CNS equivalent per well, and dilution of the first antibody 1:5000 were used.

2.6. Metabolic rate determination

The Li-7000 CO_2/H_2O analyser (Li-COR Biosciences, Lincoln, NE, USA) was used to measure the rate of carbon dioxide production - a marker of the total metabolic level -according to a protocol published elsewhere (Kodrík et al., 2010). Eight individual cockroaches were measured separately in eight measuring chambers for a period of 40 min. Data were analysed by data acquisition software (Sable System, Las Vegas, Nevada, USA). The carbon dioxide production (VCO_2) was calculated from fractional concentrations of carbon dioxide going in (FI) and coming out (FE) of the respirometry chamber using an equation according to Withers (1977) and expressed in µl min^{-1} $adult^{-1}$ units: $VCO_2 = (FECO_2 - FICO_2)\,f$, where f is the flow rate in µl h^{-1}.

2.7. Level of energy substrates

The levels of lipids and carbohydrates were determined in the *P. americana* haemolymph 24 h after the *I. fumosorosea* and Peram-CAH-II treatments. Before processing, the haemocytes were removed from haemolymph samples by centrifuging at 1000 g for 2 min at 4 °C, and 1 µl of supernatant per sample was used for determination of the lipids and carbohydrates.

- *Lipid determination* – was done by sulpho-phosho-vanillin method according to Zöllner and Kirsch (1962), as modified by Kodrík et al. (2000). The optical densities at 546 nm, measured in a spectrophotometer (UV 1601 Shimadzu), were converted to µg lipids per µl haemolymph with the aid of a calibration curve based on known amounts of oleic acid.
- *Free carbohydrate determination* – the haemolymph supernatant was diluted hundred times in distilled water and resulting solution used for quantification of free carbohydrate level by the anthrone method (Carroll et al., 1956) as modified by Socha et al. (2004). The glucose standard curve was used for the carbohydrate quantification.

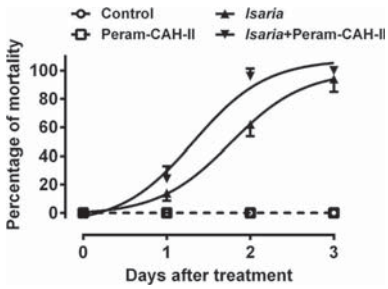

Fig. 3. The effect of *I. fumosorosea* blastospore (500,000/ind.) and Peram-CAH-II (40 pmol/ind.) injections on mortality of *P. americana* adult males 1, 2 and 3 days after the treatments. Repeated measures ANOVA with the Tukey's post-test revealed significant differences among all treatments with the exception of control vs. Peram-CAH-II at 1% level; n = 4–5 groups with 20 individuals in each.

2.8. Catalase activity in midgut

To determine the activity of catalase the midgut was homogenized in cold phosphate buffer (pH 7.0) in a ratio of 30 µl buffer per 1 mg fresh midgut weight. The homogenate was centrifuged for 15 min at 7000 *g* at 4 °C, and the supernatant used for the assay. The assay employed the Amplex Red Catalase Assay Kit (Molecular Probes, A22180) and was performed according to the instructions given in the manufacturer protocol.

2.9. Data presentation and statistical analyses

The results were plotted using the graphic software Prism (Graph Pad Software, version 6.0, San Diego, CA, USA). The points in the line graphs, and bars in the bar graphs represent mean ± SD, the numbers of replicates (n) are depicted in the figure legends. Data were analysed by repeated measures ANOVA (Figs. 1–3) (since percentage data are not normally distributed, the arcsine square-root transformation (Sokal and Rohlf, 1969; Zar, 1999) was used to normalize them before statistical analyses), by one-way ANOVA with Tukey's post-test (Figs. 5–7), and by Student's t-test (Fig. 4), using the Prism software.

3. Results

3.1. Effect of I. fumosorosea on cockroach mortality

In the first series of experiments, we determined the optimal dose of *I. fumosorosea* blastospores that elicited relevant stress after application, but left a sufficient amount of living cockroaches for subsequent analyses. The effect of various doses of blastospores on cockroach mortality is shown in Fig. 2. The results revealed increasing mortality after application of increasing doses of blastospores, and the highest dose (500,000 per cockroach) resulted in almost 100% mortality within 3 days. Thus, we used this dose for the subsequent experiments and evaluated the effect 1 and 2 days after treatment. The co-application of *I. fumosorosea* blastospores with Peram-CAH-II (40 pmol) significantly increased cockroach mortality (Fig. 3) by approximately 1.7- and 1.5-fold (compared with *I. fumosorosea* alone) 1 and 2 days after treatment, respectively. The control injection of Ringer saline as well as application of Peram-CAH-II had no effect on mortality.

3.2. Effect of I. fumosorosea on AKHs in CNS

I. fumosorosea application is considered to cause severe stress in the cockroach body, and thus activates defence reactions that include mobilisation of the endocrine and nervous systems and corresponding physiological and biochemical reactions. This prerequisite was evaluated based on *Peram-Cah-II* gene expression and levels of Peram-CAH-I and -II peptides in the cockroach CNS. The results revealed significant increases in *Peram-Cah-II* gene expression—2.1- and 1.7-fold—1 and 2 days, after *I. fumosorosea* treatment, respectively (Fig. 4A). Correspondingly, the levels of Peram-CAH-I and -II peptides significantly increased by 2.7- and 1.9-fold 1 and 2 days after treatment, respectively, compared with the relevant controls (Fig. 4B).

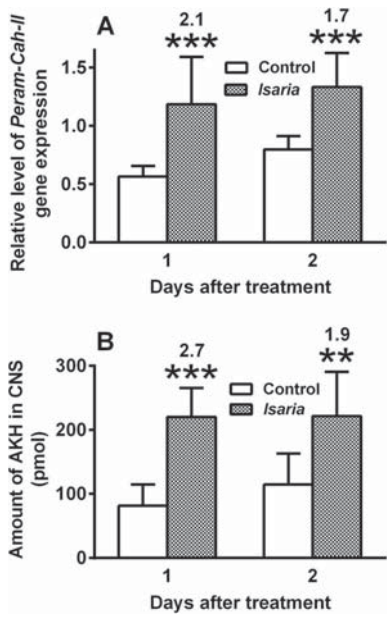

Fig. 4. The effect *I. fumosorosea* blastospore (500,000/ind.) injection on (A) *Peram-Cah-II* gene expression, and on (B) total AKH amount in *P. americana* adult male CNS 1 and 2 days after the treatment. Statistically significant differences between the *I. fumosorosea* treated groups and untreated controls at the 0.1% and 1% levels evaluated by Student's t-test are indicated by *** and **, respectively. The numbers above the columns represent fold-differences of *Peram-Cah-II* gene expression (A) and AKH level (B) in the *I. fumosorosea* treated groups as compared with untreated controls; bars = mean ± SD, *n* = 6–8.

3.3. Effect of I. fumosorosea on metabolic characteristics

We first tested the hypothesis that external application of AKH increases mortality induced by toxic factors by simple stimulation of total metabolism, resulting in higher turnover of metabolites, including toxins and thus faster penetration of toxins into tissues and cells (Kodrík et al., 2010). Carbon dioxide production by the cockroaches was monitored as an indicator of metabolic intensity. Although the application of *I. fumosorosea* or Peram-CAH-II slightly but insignificantly stimulated carbon dioxide production, co-application of *I. fumosorosea* and Peram-CAH-II led to a significant increase (1.6-fold) in carbon dioxide production compared with that after application of *I. fumosorosea* alone (Fig. 5).

The hypothesis, that has been tested, is the assumption that increased nutrient levels in the haemolymph caused by AKH, provide a

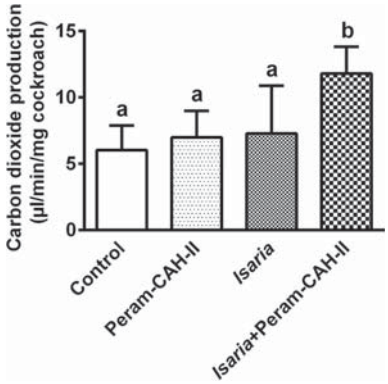

Fig. 5. The effect of *I. fumosorosea* blastospore (500,000/ind.) and Peram-CAH-II (40 pmol/ind.) injections on carbon dioxide production in *P. americana* adult males 1 day after the treatments. Statistically significant differences among the experimental groups at the 0.1% level evaluated by one-way ANOVA with the Tukey's post-test are indicated by different letters; bars = mean ± SD, n = 12–13.

higher-quality substrate for the propagation of toxic pathogens in the insect body (Mullen and Goldsworthy, 2006). Levels of energy-related nutrients—carbohydrates and lipids—were monitored after the *I. fumosorosea* and hormonal treatments. The results revealed a significant increase (about 2.3-fold) of free carbohydrate levels in cockroach haemolymph treated with Peram-CAH-II and a significant decrease (about 1.4-fold) after *I. fumosorosea* application (Fig. 6A). The co-application of *I. fumosorosea* and the hormone significantly increased the carbohydrate level in the haemolymph by 1.8- and 1.3-fold, compared to *I. fumosorosea* treatment and control levels, respectively. The hormonal treatment had no effect on the lipid level in cockroach haemolymph (Fig. 6B); however, the lipid level significantly decreased after the *I. fumosorosea* treatment (approximately 2.0-fold), and, as in the case of free carbohydrates, significantly increased (approximately 1.8-fold) after *I. fumosorosea* and Peram-CAH-II co-application, compared to that in controls.

The effect of possible oxidative stress after the *I. fumosorosea* treatment was monitored by catalase activity in the cockroach gut (Fig. 7). The results showed significantly decreased catalase activity after *I. fumosorosea* application (approximately 2.2-fold) and significantly increased activity after co-treatment with *I. fumosorosea* and Peram-CAH-II (approximately 3.6- and 1.6-fold, compared with that of the *I. fumosorosea* treatment and control, respectively). Application of the hormone alone had no effect.

4. Discussion

The fungus *I. fumosorosea* is a well-known entomopathogenic species frequently used for practical applications, and thus its biology is well understood (Zimmermann, 2008). Especially, the strain CCM 8367, used in this study, was found earlier to be highly virulent against various pest species (Hussein et al., 2013, 2016) and is promising also for preventive application to soil or substrate (Zemek et al., 2018). *I. fumosorosea* usually initiates infection in the insect host by conidial adhesion to the cuticle followed by germination, development of appressoria and penetration of hyphae through the cuticle. In the host haemocoel, the hyphae transform into unicellular thin-wall blastospores that lack immunogenic properties (Butt et al., 2016) and propagate rapidly by budding in the haemolymph and tissues utilising

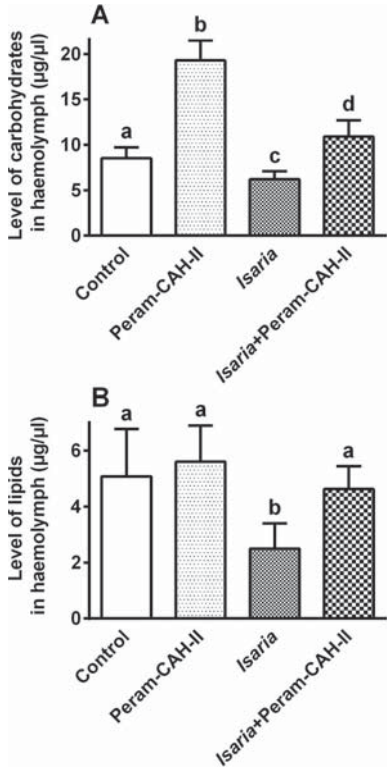

Fig. 6. The effect of *I. fumosorosea* blastospore (500,000/ind.) and Peram-CAH-II (40 pmol/ind.) injections on carbohydrate (A) and lipid (B) levels in *P. americana* adult male haemolymph 1 day after the treatment. Statistically significant differences among the experimental groups at the 5% level evaluated by one-way ANOVA with the Tukey's post-test are indicated by different letters; bars = mean ± SD, n = 8–11 (A) and n = 8–14 (B).

the host nutrients. Infected insects commonly die within a few days owing to invasion of the blastospores into tissues and cells and their destruction as well as nutritional depletion and total mummification of the host (Pendland et al., 1993; Zhu et al., 2016). Further, the fungi produce a wide spectrum of enzymes, toxins and other biologically active compounds during their development to facilitate the infection (Luangsa-Ard et al., 2009). Infected insects activate their defence systems to reduce the impact of the infection on homeostasis and biological functions; both humoral and cellular immune responses are activated (Lu and St Leger, 2016). Firstly, the invading fungi are detected by recognition molecules in insect body that switch on the general immune responses via the Imd and Toll pathways (Valanne et al., 2011). Further, the humoral immunity employs mainly antimicrobial peptides (AMPs), a wide range of very effective small peptidic molecules, produced in the fat body (Fehlbaum et al., 1994). Cellular response involve haemocytes responsible for phagocytosis, nodulation and encapsulation (Strand, 2008). Additionally also coagulation and melanisation of haemolymph controlled by proteolytic cascades may be involved (Cerenius et al., 2010). AKHs play an important role in this pathogen-elicited stress, as they are responsible for activation of anti-

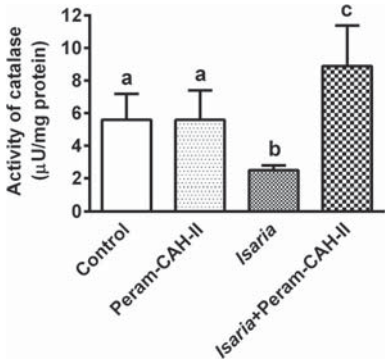

Fig. 7. The effect of *I. fumosorosea* blastospore (500,000/ind.) and Peram-CAH-II (40 pmol/ind.) injections on catalase activity in *P. americana* male midgut 1 day after the treatment. Statistically significant differences among the experimental groups at the 1% level evaluated by one-way ANOVA with the Tukey's post-test are indicated by different letters; bars = mean ± SD, n = 5–7.

stress responses (Kodrík, 2008). Accordingly, we observed significant increases in both *Peram-Cah-II* gene expression, and Peram-CAH-I and -II peptide levels after *I. fumosorosea* application.

Although AKHs are known as anti-stress hormones, a series of studies recently demonstrated that co-application of AKH with various toxins/insecticides (Kodrík et al., 2010; Velki et al., 2011; Plavšin et al., 2015) or the entomopathogenic nematode *Steinernema carpocapsae* (Ibrahim et al., 2017, 2018) dramatically increases the efficacy of the toxic agents as indicated by increased mortality. A similar phenomenon was reported for the gram-positive bacterium *Bacillus megaterium* and fungus *M. anisopliae* (e.g. Goldsworthy et al., 2002a, 2003a, 2003b; Mullen and Goldsworthy, 2006). In this study, we found that application of blastospores of the fungus *I. fumosorosea* to the cockroach, *P. americana* produced the same effect. The mechanisms underlying this behaviour of AKH are not completely clarified, but there are three possible explanations that were at least partially supported by the results.

First of all, the main function of AKHs is mobilisation of energy—lipids, carbohydrates, and/or proline (Gäde et al., 1997)—under metabolic stress. During this process, total body metabolism is elevated, which may intensify metabolite turnover. If any toxins are present, they might penetrate cells faster, which may increase their efficacy and impact on insect physiological and biochemical processes. These events might occur before the defence reactions in the body are activated. Correspondingly, in this study, a significant increase in carbon dioxide production as a marker of metabolic intensity was observed after Peram-CAH-II and *I. fumosorosea* co-application (see Fig. 5). This is in agreement with previous findings obtained after the co-application of insecticides (Kodrík et al., 2010; Velki et al., 2011; Plavšin et al., 2015) or nematodes (Ibrahim et al., 2017, 2018) and AKH. Only a slight and insignificant elevation of carbon dioxide production after application of AKH alone was observed in these previous studies and the current study. The AKH response is suggested to be fully developed only when a stressor is present. Numerous examples to support this statement can be found in literature. For instance, the defence response stimulated by Locmi-AKH-I injection in the migratory locust (*L. migratoria*) was provoked only when immunogen was present (Goldsworthy et al., 2002a). Further, the stimulation of locomotor activity by Pyrap-AKH was positively time-correlated with the hyperlipaemic effect in the firebug *Pyrrhocoris apterus* only when the hormone was applied using an injection associated with injury (that served as the stressor); when Pyrap-AKH was applied topically, the time correlation between the both AKH responses was not recorded (Kodrík et al., 2002).

The second possible explanation of the increased toxin efficacy due to AKH was previously hypothesised by Mullen and Goldsworthy in 2006. The authors suggested that nutrient elevation in the haemolymph after AKH application may support pathogen proliferation, and thus increase virulence. In accordance with this hypothesis, we observed significantly increased free carbohydrate levels in the haemolymph after the Peram-CAH-II treatment. This is not surprising, because the hypertrehalosemic effect of this hormone in the American cockroach is well established (Scarborough et al., 1984). The application of *I. fumosorosea* lowered the level of carbohydrates possibly due to their utilisation for fungal metabolism and development. The co-application of *I. fumosorosea* and the hormone significantly increased the carbohydrate level; however, the increase was not as high after the injection of the hormone alone. On the other hand, the metabolism of lipids seems to be more complicated in *P. americana* (Michitsch and Steele, 2008): injection of Peram-CAH-II does not induce mobilisation of lipids to meet energy demands, but is responsible for liberation of fatty acids from haemolymph phospholipids and their transport into the fat body, where they are converted into triacylglycerol for storage. We also found that Peram-CAH-II had no hyperlipaemic effect, but observed similar decreases and increases in lipid levels after the application of *I. fumosorosea* and co-application of *I. fumosorosea* and hormone, respectively, as observed for carbohydrates (see Fig. 6). Nonetheless, the cause of this behaviour of AKH remains unclear, especially in light of the findings of Michitsch and Steele (2008).

The final possible explanation of the stimulatory effect of AKH on toxin efficacy is related to its general inhibition of protein synthesis (Carlisle and Loughton, 1979; Kodrík and Goldsworthy, 1995). AKH activity can reduce the synthesis of antimicrobial peptides (AMPs), which are mostly small peptides produced by the fat body or haemocytes that are thought to function by disrupting bacterial membranes (Lemaitre and Hoffmann, 2007). Dozens of AMPs have been characterised in insects (Beckage, 2008), and new AMPs continue to be discovered; in *Drosophila melanogaster*, at least 30 AMPs have been predicted from genome analysis (Adams et al., 2000). The mechanism by which AKHs could restrict the synthesis of AMPs is not known, but either direct blocking of peptide synthesis or inhibition of RNA synthesis (Kodrík and Goldsworthy, 1995) is possible.

In the present study, *I. fumosorosea* significantly impaired catalase activity in the cockroach's gut. Catalase is one of the key enzymes of the anti-oxidative stress response that converts relatively toxic hydrogen peroxide to harmless water and oxygen. Generally, oxidative stress is generated when the production of free radicals/reactive oxygen species, as products of normal oxygen metabolism, is enhanced and their scavenging systems are reduced. Higher levels of reactive oxygen species damage DNA, proteins, and lipid molecules, injure cell membranes, and cause uncontrolled apoptosis. On the other hand, reactive oxygen species, at physiological levels, are utilised in immune defence reactions against bacteria and other pathogenic microorganisms (Slauch, 2011). Catalase activity is either reduced (Večeřa et al., 2012; Bednářová et al., 2013) or enhanced (Velki et al., 2011; Plavšin et al., 2015) after the application of chemical oxidative stressors, depending on the reaction in the scavenging systems of the reactive oxygen species affected by the stressor. AKHs are known to stimulate anti-oxidative stress reactions (for review, see Kodrík et al., 2015) and affect catalase activity: according to the need, they either increase or decrease the catalase activity when disturbed by stressors (Kodrík et al., 2015). Thus, the reduction in catalase activity in the cockroach gut after the *I. fumosorosea* treatment observed in this study might have resulted from over-production of reactive oxygen species that impaired catalase, perhaps by protein carbonylation. On the other hand, targeted elimination of cockroach catalase by *I. fumosorosea* as part of an anti-oxidative defence system cannot be excluded. In accordance with the

literature mentioned above, the application of Peram-CAH-II restored the catalase activity reduced by *I. fumosorosea*; nevertheless, the mechanism is likely more complicated and therefore requires additional investigation. Further, the lack of effect of Peram-CAH-II alone on the catalase activity demonstrated that AKH action can be fully realised merely in the presence of a stressor as discussed above.

In conclusion, the present study demonstrates that the entomopathogenic fungus *I. fumosorosea* induces severe stress in the body of the cockroach, *P. americana* as indicated by the stimulation of AKH expression. The effect of *I. fumosorosea* was enhanced by the external application of Peram-CAH-II. The mechanism of AKH action could involve (1) stimulation of metabolism (indicated by increased production of carbon dioxide), which can increase the turnover of toxins produced by *I. fumosorosea* in the cockroach's body; (2) enrichment of the haemolymph with nutrients (indicated by mobilisation of carbohydrates); and (3) inhibition of synthesis of AMPs (hypothesised). *I. fumosorosea* and Peram-CAH-II probably also affect oxidative stress through the modification of catalase activity in the cockroach's gut, however, the mechanism is unclear. Our study represents basic research, but the results might be useful for the development of future strategies of biological control of insect pests. Therefore, detailed studies including the effects on *P. americana* nymphs and females are also needed.

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Declaration of competing interest

There are no conflicts of interest to declare.

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5.2. Paper II

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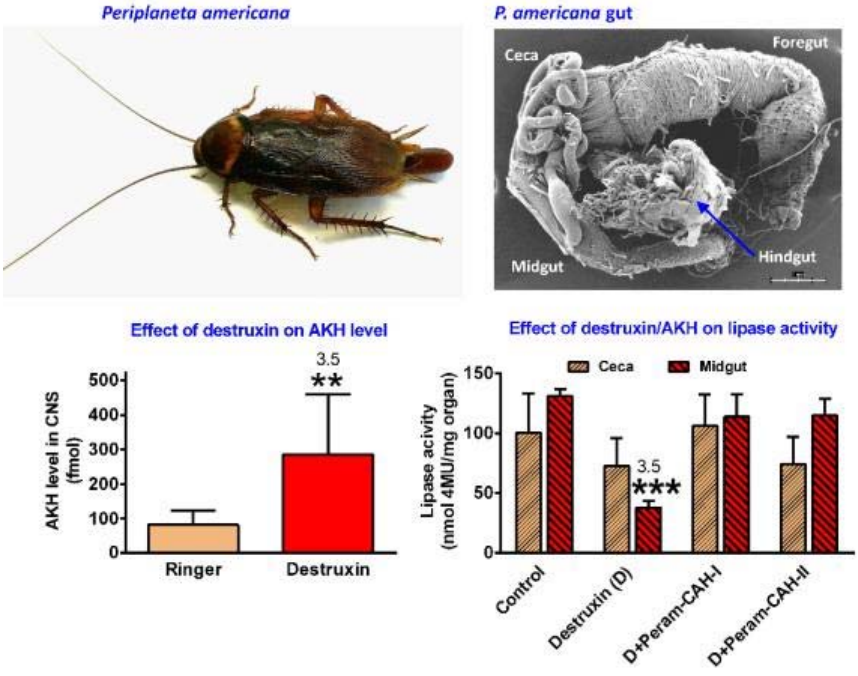

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RESEARCH ARTICLE

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Effect of natural toxins and adipokinetic hormones on the activity of digestive enzymes in the midgut of the cockroach *Periplaneta americana*

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Abstract

This study examined the effect of two natural toxins (a venom from the parasitic wasp *Habrobracon hebetor* and destruxin A from the entomopathogenic fungus *Metarhizium anisopliae*), and one pathogen (the entomopathogenic fungus *Isaria fumosorosea*) on the activity of basic digestive enzymes in the midgut of the cockroach *Periplaneta americana*. Simultaneously, the role of adipokinetic hormones (AKH) in the digestive processes was evaluated. The results showed that all tested toxins/pathogens elicited stress responses when applied into the cockroach body, as documented by an increase of AKH level in the central nervous system. The venom from *H. hebetor* showed no effect on digestive enzyme activities in the ceca and midgut in vitro. In addition, infection by *I. fumosorosea* caused a decrease in activity of all enzymes in the midgut and a variable decrease in activity in the ceca; application of AKHs did not reverse the inhibition. Destruxin A inhibited the activity of all enzymes in the midgut but none in the ceca in vitro; application of AKHs did reverse this inhibition, and no differences between both cockroach AKHs were found. Overall, the results demonstrated the variable effect of the tested toxins/pathogens on the digestive processes of cockroaches as well as the variable ability of AKH to counteract these effects.

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KEYWORDS
AKH, amylase, insect digestion, lipase, midgut enzymes, natural toxins, protease

1 | INTRODUCTION

It is well known that various toxins, pathogenic organisms as well as physical and physiological stressors may upset functional homeostasis in the animal body (Ivanovič & Jankovič-Hladni, 1991). All these stressors usually elicit the typical reaction of the organism, which is often referred to as a generalized defense response. This response includes various processes occurring from the cellular to organismal levels in the animal body (Hightower, 1991). The defense reactions are controlled by the nervous and endocrine systems. The nervous system is responsible for rapid, immediate reactions, while the endocrine system provides deeper, usually long-term (from minutes to days) changes. To combat these stressors, animals have developed a variety of antistress hormones. In insects, the antistress reactions seem to be regulated predominantly by adipokinetic hormones (AKHs) released from the corpora cardiaca, a small neuroendocrine gland associated with the brain. AKHs functionally resemble mammalian glucagon (Alquicer, Kodrík, Krishnan, Večeřa, & Socha, 2009; Bednářová, Kodrík, & Krishnan, 2013) but their structure is different: they consist of only 8–10 amino acids (Gäde, Hoffmann, & Spring, 1997). Generally, these hormones are a typical example of neuropeptides, with complex functions in the control of insect metabolism, primarily in the mobilization of different kinds of energy reserves such as lipids, carbohydrates, and/or, certain amino acids (Gäde & Goldsworthy, 2003; Gäde et al., 1997). However, AKHs are pleiotropic in nature and are involved in many metabolic activities including various aspects of the defense and antistress responses (Gäde et al., 1997; Kodrík, 2008). Thus, AKHs enhance food intake and digestive processes in the insect gut (Bil, Broeckx, Landuyt, & Huybrechts, 2014; Bodláková, Beňová, & Kodrík, 2018; Kodrík, Vinokurov, Tomčala, & Socha, 2012), stimulate general locomotion (Socha, Kodrík, & Zemek, 1999), participate in the activation of antioxidant mechanisms (Kodrík, Bednářová, Zemanová, & Krishnan, 2015; Kodrík, Krishnan, & Habuštová, 2007), and interact with the cellular and humoral immune systems (Goldsworthy, Opoku-Ware, & Mullen, 2002).

AKHs have been shown to induce defense reactions of the insect body against natural toxins or pathogens. For example, AKH was found to significantly reduce the toxicity of the venom from the parasitic wasp *Habrobracon hebetor* (Shaik, Mishra, & Kodrík, 2017). This venom elicits complete neuromuscular paralysis of the locomotory apparatus in insects (Beckage & Gelman, 2004) and strongly suppresses cell and humoral immunity (Kryukova et al., 2011; Pennacchio, Caccia, & Digilio, 2014). Although the chemical composition of the venom is not well understood, it is established that the venom is a complex cocktail of mainly proteinaceous compounds ranging from 18 to 73 kDa. There have been several attempts to isolate the responsible toxin(s) but variable results suggest the presence of more active components or cooperating activities among these components in the host body (Slavnova et al., 1987; Visser, Labruyere, Spanjer, & Piek, 1983). In the firebug *Pyrrhocoris apterus*, the application of this venom significantly stimulated *Akh* gene expression and enhanced the AKH level in the insect's body; correspondingly, the external application of AKH significantly reduced the paralyzing effect of the venom on this bug (Shaik et al., 2017). Furthermore, the prophenoloxidase cascade in the hemolymph of the locust *Locusta migratoria* was activated when laminarin (an algal-derived immunogen fairly typical of the β-1,3-glucans present in fungal cell walls) was injected, and this activation was prolonged when locust AKH (Locmi-AKH-I) was coapplied with laminarin (Goldsworthy, Opoku-Ware, et al., 2002). Additionally, injected AKH affected the impact of the Gram-positive bacterium *Bacillus megaterium* injected into the locust body as well as topically applied conidia of the entomopathogenic fungus *Metarhizium anisopliae* (Goldsworthy, Opoku-Ware, & Mullen, 2005). A cyclodepsipeptide mycotoxin called destruxin A is responsible for the toxicity of the latter fungus. Its structure has been elucidated in the past (Suzuki, Kuyama, Kodaira, & Tamura, 1966) and the pure compound is commercially available (Sigma-Aldrich). Entomopathogenic fungi occur widely; some of these are used for pest insect control, for example, *Isaria fumosorosea*, which was used in the present study (Prenerová, Zemek, Weyda, &

Volter, 2009). An insecticidal depsipeptide was identified in this fungus (Luangsa-Ard, Berkaew, Ridkaew, Hywel-Jones, & Isaka, 2009); however, it is known rather from another fungus *Beauveria bassiana*, from which the name of this toxin, beauvericin, was derived. Beauvericin is responsible for eliciting membrane disruption and cell permeabilization (Mallebrera, Prosperini, Font, & Ruiz, 2018) in infected insects; however, the mechanisms are not well understood.

The main goal of this study was to examine the role of AKHs in regulating the activity of basic digestive enzymes in the gut of the American cockroach *Periplaneta americana* following treatment with two natural toxins and one entomopathogenic organism. Mutual relationships between the toxins/pathogen and hormones were also evaluated.

2 | MATERIALS AND METHODS

2.1 | Insects

A mass culture of the American cockroach *P. americana* used in the present study was kept in 60 L glass fish tanks under short-day conditions (12-hr light:12-hr dark) at a constant temperature of 30 ± 1 °C. The cockroaches were supplied with food (apples, carrots, stale bread, and oat flakes) and water ad libitum. As there were found no differences in the studied parameters between sexes (Bodláková et al., 2018), adults of unknown age were used for the experiments.

2.2 | Application of toxic agents and AKHs: Gut experiments

2.2.1 | The effect of *H. hebetor venom* and destruxin toxin (in vitro experiments)

The gastric ceca and midgut were dissected from the cockroaches, washed in saline, and incubated for 24 hr in 500 μl of Grace's medium (the amount corresponding with estimated body volume; see Bodláková et al., 2018) at 30°C. Two tested toxins—a venom from a parasitic wasp *H. hebetor* or toxin destruxin A from *M. anisopliae* was added into the medium. The *H. hebetor* venom was extracted from the adult female venom sacs by Ringer saline (100 sacs were homogenized in 1 ml Ringer saline) and filtered through 0.22 μM filters (for details see Shaik et al., 2017). A dose of 1 sac equiv. (in 2 μl) selected in initial experiments (data not shown) per organ (ceca or midgut) was used in the experiments. The destruxin A was purchased from Sigma-Aldrich company; its 50 nmol equiv. in Ringer saline (in 2 μl) was used for the in vitro experiments. The dose was selected in initial experiments similarly as mentioned for the *H. hebetor* venom. For both toxins, the corresponding controls were carried out; in them, only the corresponding volume of the solvent (Ringer saline) was applied into the medium. Twenty-four hours later the organs from both experimental and control incubations were taken out from the medium, carefully dried, weighed and stored at −20°C until use for enzyme activity determination (see below).

2.2.2 | The effect of *I. fumosorosea* infection (in vivo experiments)

In these experiments, the effect of entomopathogenic fungus *I. fumosorosea* on the cockroach ceca and midgut characteristics was studied. The *I. fumosorosea*, (strain number CCM8367; Prenerová et al., 2009) was obtained by courtesy of Dr. A. Bohatá (Agricultural Faculty, South Bohemian University, České Budějovice). The fungus blastospores were prepared in potato dextrose broth and their concentration optimized with hemocytometer Neubauer Chamber (Marienfeld, Germany). For the infection, the cockroaches were injected with a dose of 500,000 blastospores/individual (selected in initial experiments—data not shown) in 5 μl Ringer saline; controls were injected by the Ringer saline only. All the cockroaches had access to food. Twenty-four hours later, the experiment was terminated with the ceca and midgut dissection. The organs were dried, weighed and stored at −20°C until used for enzyme activity determination (see below).

2.2.3 | Hormonal treatment

The used cockroach AKHs Peram-CAH-I and Peram-CAH-II (Scarborough et al., 1984) were commercially synthesized in the Polypeptide Laboratories, Praha. They are both octapeptides that differ in three amino acids—Peram-CAH-I: pGlu-Val-Asn-Phe-Ser-Pro-Asn-Trp-NH_2 and Peram-CAH-II: pGlu-Leu-Thr-Phe-Thr-Pro-Asn-Trp-NH_2. Those AKHs, in a dose of 40 pmol (Bodláková, Jedlička, & Kodrík, 2017), were either pipetted into the incubation medium (in 2 μl) or injected into the cockroach bodies (in 2 μl) to monitor their impact on intoxified cecas or midguts. Thus, the usual design of the experiments was: (a) control, (b) toxin/pathogen alone treatment, (c) toxin/pathogen + Peram-CAH-I treatment, and (d) toxin/pathogen + Peram-CAH-II treatment. The effect of the hormones only was omitted, because this topic was studied in our previous paper (Bodláková et al., 2018).

2.3 | Enzyme activity determinations

The activities of proteases, lipases, and amylases were determined in the gastric ceca and midgut. The organs were homogenized (sonicated) in the appropriate buffer (see below), centrifuged and the aliquot 0.005 equiv. tested for enzyme activity.

2.3.1 | Protease assay

The protease activity was assessed with the resorufin-casein kit (Roche) according to manufacturer's instructions. Briefly, 20 μl sample extracts in 0.2 M tris pH 7.8 were mixed with 20 μl of 0.4% substrate (resorufin-casein) and 20 μl of 0.02 M calcium chloride solution, and adjusted up to 100 μl by using the same tris buffer in the microtubes. The mixture was subsequently subjected to gentle shaking for 1 hr at 37°C. The reaction was terminated by addition of 240 μl of 5% trichloroacetic acid, and after 10 min of subsequent incubation at 37°C centrifuged to remove the nonhydrolyzed casein. The absorbance was measured at 490 nm. Appropriate controls without the samples were assayed simultaneously. Protease activity was expressed in units of proteolytic activity per mg of fresh organ weight; this unit (U) was defined as the amount of enzyme (mg) which caused an increase in optical density by 0.1 per min in 1 ml of the reaction mixture (Elpidina et al., 2001).

2.3.2 | Lipase assay

The lipase activity was assessed with 4-methylumbelliferyl butyrate (4-MU butyrate) according to Roberts (1985) as modified by Kodrík et al. (2012). Five microliters of 2 mM substrate diluted in dimethylsulfoxide (DMSO) were added to microplate wells with organ extracts in 0.2 M tris pH 7.8 diluted to 195 μl with 100 mM phosphate buffer pH 5.0 (Frugoni, 1957). Samples were incubated at 30°C and the release of the fluorescent 4-methylumbelliferone (4-MU) was monitored at 5 min intervals at 327 nm excitation and 449 nm emission with a Synergy 4 multimode microplate reader (BioTek Instr., Winooski, Vermont). Activity was expressed in nanomoles of 4-MU·min^{-1}·mg^{-1} of fresh organ weight.

2.3.3 | Amylase assay

The assay was performed with 3,5-dinitrosalicylic acid reagent (DNS) according to Bernfeld (1955) as modified by Kodrík et al. (2012). Briefly, a 25 μl aliquot sample extract in 100 mM phosphate buffer (Frugoni, 1957) pH 5.7 was mixed with an equal volume of 2% soluble starch made of the same buffer with 40 mM NaCl. The reaction mixture was incubated at 30°C and under constant agitation for 40 min until it was terminated by adding 200 μl DNS. Then the solution was heated at 100°C for 5 min, cooled, clarified by centrifugation (10,000*g* for 10 min), and the

absorbance was read in supernatant at 550 nm. Enzyme activity was calculated in micromoles maltose per milligram of fresh organ weight.

2.4 | Extraction of AKHs from CNS and determination of their level by enzyme-linked immunosorbent assay (ELISA)

In some experiments, the effect of the toxic agents on AKH level in the central nervous system (CNS) was evaluated. For that, a dose of 1 venom sac equiv. from *H. hebetor*, 500,000 blastospores of *I. fumosorosea* or 50 nmol destruxin A (see also above) was injected into the cockroach body; controls were injected by the Ringer saline only. Twenty-four hours later, CNS containing the brain with corpora cardiaca attached was dissected from the cockroach head cut off from the body under the Ringer saline. The AKHs were extracted from the CNS using 80% methanol and the solution was evaporated in a vacuum centrifuge. The resulting pellet was used in competitive ELISA for the AKH quantification. The used antibody was originally raised commercially against Cys^1-Pyrap-AKH (Sigma Genosys, Cambridge, UK), however, it recognized well both *P. americana* AKHs: Peram-CAH-I and -CAH-II (Goldsworthy, Kodrík, Comley, & Lightfoot, 2002). A biotinylated probe was prepared from Cys^1-Pyrap-AKH using Biotin Long Arm Maleimide (BLAM, Vector Laboratories, Peterborough, UK). The ELISA comprised precoating of the 96-well microtiter plates (high-binding Costar; Corning Incorporated, Corning, NY) overnight with the antibody preparation in coating buffer. After blocking (with nonfat dried milk), test samples (1 CNS equiv.) were added to specific wells, followed by the biotinylated probe, both in an assay buffer. After the competition for the binding sites on the antibody bound to the plates, a streptavidin conjugated with horseradish peroxidase solution (Vector Laboratories) diluted 1:500 in phosphate-buffered saline-Tween was added to each well. All of the above mentioned steps were terminated by washing. Finally, the ELISA substrate (3,3′,5,5′-tetramethylbenzidine; Sigma-Aldrich) was added and then the reaction was stopped by adding 0.5 M sulphuric acid. The absorbance values were determined in a microtiter plate reader at 450 nm. One row of each plate always contained a dilution series of synthetic Peram-CAH-II, which allowed the construction of a competition curve and estimation of the AKH content of unknown samples.

2.5 | Statistical analyses

The results were plotted using the graphic software Prism (Graph Pad Software, version 6.0; Graph Pad, San Diego, CA). The bar graphs represent mean ± standard deviation, the numbers of replicates (n) are depicted in the figure

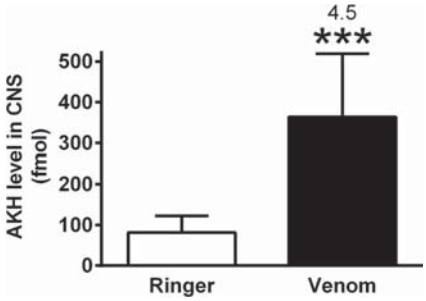

FIGURE 1 Quantification of adipokinetic hormone (AKH) level in central nervous system (CNS) of *Periplaneta americana* adults 24 hr after the *Habrobracon hebetor* venom injection (1 gland equiv. in 2 μl); controls were injected by Ringer saline only. The number above the venom column represents fold difference of AKH level as compared with control. Statistically significant difference between the experimental and control groups evaluated using Student's *t* test at the 0.1% level is indicated by *** ($n = 5–10$)

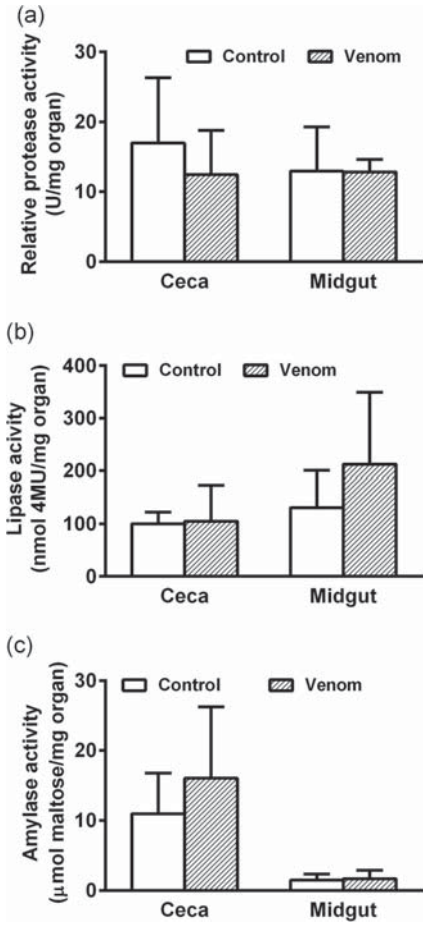

FIGURE 2 Levels of protease (a), lipase (b), and amylase (c) activities in the ceca and midgut of *Periplaneta americana* adults after 24 hr incubation in medium (500 μl) in the presence of the *Habrobracon hebetor* venom (1 gland equiv. in 2 μl); control incubations were treated by Ringer saline only. Statistical evaluation of the experimental and control groups using Student's *t* test at the 5% level revealed no significant differences ($n = 5–7$)

legends. The statistical differences were evaluated by Student's *t* test (Figures 1–3 and 5), and by one-way analysis of variance with Dunnett's posttest (Figures 4 and 6) using the Prism software (for details see Supporting Information).

3 | RESULTS

Application of various toxins is expected to cause intensive stress responses in the insect body, leading to increased activity in the hormonal and nervous systems that control the defense reactions of the body. In particular, the AKHs responsible for maintaining physiological and biochemical homeostasis in the insect body, are assumed to be activated (Kodrík, 2008). Indeed, the level of AKHs in the CNS increased by approximately 4.5 times within 24 hr

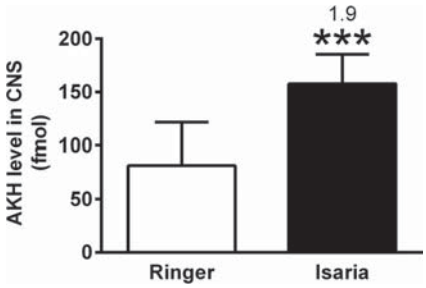

FIGURE 3 Quantification of adipokinetic hormones (AKH) level in central nervous system (CNS) of *Periplaneta americana* adults 24 hr after the *Isaria fumosorosea* injection (500,000 blastospores in 5 μl); controls were injected by Ringer saline only. The number above the *Isaria* column represents fold difference of AKH level as compared with control. Statistically significant difference between the experimental and control groups evaluated using Student's *t* test at the 0.1% level is indicated by *** ($n = 8–10$)

after injection of *H. hebetor* venom into the cockroach body (Figure 1). Interestingly, the venom application elicited no in vitro changes in the digestive enzyme activities in the gut. Neither in the ceca nor in the midgut was a significant change recorded in the activity of proteases (Figure 2a), lipases (Figure 2b), or amylases (Figure 2c).

Infection of the cockroach body by the fungus *I. fumosorosea* resulted in a significant 1.9-fold increase in the AKH level in the CNS (Figure 3). The infection also affected the activity of digestive enzymes; however, this effect was not uniform (Figure 4). The fungus significantly decreased activity of all three studied enzymes in the midgut, while the detrimental effect in the ceca was not so obvious: only the activity of amylases was significantly reduced in this organ (Figure 4c), perhaps due to the much higher level of amylase activity in the ceca than in the midgut. Despite the significant stimulatory effect of both Peram-CAH-I and Peram-CAH-II on the activity of digestive enzymes in the *P. americana* midgut and ceca (in vitro as well as in vivo) found in our previous papers (Bodláková et al., 2017, 2018), no apparent increase was recorded in the activity of those enzymes in response to AKH in the gut treated with *Isaria* (Figure 4).

The toxin destruxin A also undoubtedly stressed the treated cockroaches, as the level of AKHs in their CNS significantly increased to about 3.5 times the baseline level (Figure 5). This toxin substantially inhibited the activity of proteases (Figure 6a), lipases (Figure 6b), and amylases (Figure 6c) in the midgut; nevertheless, digestive processes in the ceca were not significantly affected by destruxin and the enzyme levels were comparable with controls. Importantly, coapplication of destruxin with both AKHs resulted in a restoration of enzymatic activity in the midgut, demonstrating the protective effect of these hormones against this toxin.

4 | DISCUSSION

Insect digestion is thought to be under hormonal control, and there are many neuropeptide candidates that are suggested to control various gut activities (Audsley & Weaver, 2009). Nevertheless, we have not achieved a detailed understanding of these processes. Recently, we found that the overall activity of digestive enzymes was enhanced in the *P. americana* gastric ceca and midgut by AKHs under in vivo and in vitro conditions, although the level of enhancement was not uniform (for details see Bodláková et al., 2017, 2018). This variability was reflected in different activity levels of particular enzymes in the ceca (higher activity of amylases) and the midgut (higher activity of lipases), a finding supported by this study. Results from in vitro stimulation suggests a direct hormonal action in the gut without involvement of other systems.

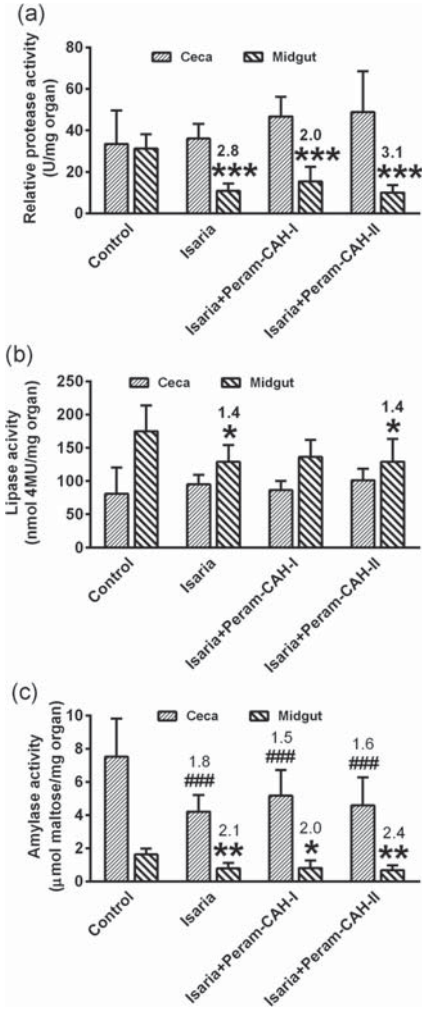

FIGURE 4 Levels of protease (a), lipase (b) and amylase (c) activities in ceca and midgut of *Periplaneta americana* adults 24 hr after injection of *Isaria fumosorosea* blastospores (500,000 in 5 μl), Peram-CAH-I (40 pmol in 2 μl) and Peram-CAH-II (40 pmol in 2 μl); controls were injected by Ringer saline only. The numbers above the columns represent fold difference of *Isaria* or *Isaria* + AKH columns as compared with the corresponding control (ceca or midgut). Statistically significant differences between the experimental and control groups evaluated using one-way analysis of variance with Dunnett's post hoc test at the 0.1% level are indicated by ### (ceca) or at the 0.1%, 1% and 5% by ** and *, respectively (midgut; $n = 6–8$)

There are many insect pathogens and associated toxins that exert their effects via destabilization of insect gut functions. They may interact directly with the gut cells or indirectly via disruption of general homeostasis. A classic example of direct interactions is the group of Bt toxins, entomotoxic proteins produced by the bacteria *Bacillus thuringiensis*. Bt toxins affect the insect gut, where they dissolve, activate, and interact with specific receptors on

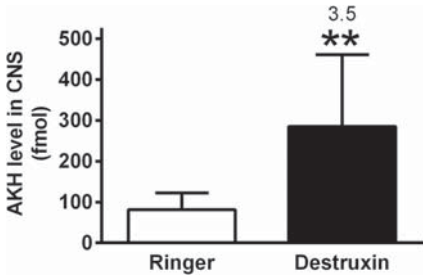

FIGURE 5 Quantification of adipokinetic hormones (AKH) level in central nervous system (CNS) of *Periplaneta americana* adults 24 hr after the destruxin A injection (50 nmol in 2 μl); controls were injected by Ringer saline only. The number above the destruxin column represents fold difference of AKH level as compared with control. Statistically significant difference between the experimental and control groups evaluated using Student's *t* test at the 1% level is indicated by **(n = 6–10)

the apical surface of the midgut epithelial cells. This leads to the formation of pores, ionic imbalance, cell lysis, and septicemia (Schnepf et al., 1998). In this study, we tested several toxins with different modes of action.

The principal natural function of *H. hebetor* venom is neuromuscular paralysis of insect skeletal muscles (Beckage & Gelman, 2004). This is utilized by adult female wasps, which sting the host and inject the venom to immobilize it and to lay eggs into its body. The detailed mechanism of the venom's action is not known but it is thought that the venom blocks receptors on the presynaptic membranes of neuromuscular junctions and inhibits exocytosis in presynaptic vesicles (Pennacchio & Strand, 2006; Weaver, Marris, Bell, & Edwards, 2001). It appears that only skeletal muscles are paralyzed, although all insect muscles including the visceral ones are striated. This assumption was recently confirmed by Sláma and Lukáš (2011), who proved that organs with myogenic control of their contraction—the heart and gut—were fully active in the venom-paralyzed larvae of the wax moth, *Galleria mellonella*. Consequently, the venom does not affect the neuronal systems regulated by a junction potential or interneuronal communication; rather, only the transmission of the neuromuscular action potential is disabled. We have found in this study that this venom might also be active in cockroaches, as its application significantly increased the AKH level in the CNS; the same effect was observed in *P. apterus* (Shaik et al., 2017). Additionally, our results support the finding of Sláma and Lukáš (2011) that the venom does not affect gut functions.

We also examined the effect of *I. fumosorosea*, an entomopathogenic fungus that starts its infection by breaching the host cuticle and penetrating into the host body. *Isaria* spp. employ a set of enzymes, proteases, amylases, lipases, chitosanases, or chitinases (Ali, Huang, & Ren, 2010; Hajek & Leger, 1994), to dissolve the host tissues and utilize them as a source of food and energy for their own growth and development. In this study, we used injection of *Isaria* blastospores to speed up the resulting infection in the cockroaches. *Isaria* treatment likely elicited an intensive stress response in the experimental cockroaches as evidenced by a significant increase in the AKH level in the brain as well as a significant decrease in digestive enzyme activity in the midgut; interestingly, in the ceca only the amylase activity was affected. Additionally, neither of the cockroach AKHs could restore midgut enzymatic activities. The mechanism underlying this phenomenon is unknown.

The third toxin tested was destruxin A. There are several types of destruxins that affect different organs (gut, Malpighian tubules, and muscles) in insects. Destruxins inhibit the hydrolytic activity of V-type H^+-ATPase of the brush border membrane vesicles (Bandani, Amiri, Butt, & Gordon-Weeks, 2001), induce opening of Ca^{++} channels in skeletal and visceral muscles (Ruiz-Sanchez, Lange, & Orchard, 2010), and activate phosphorylation of proteins in insect cell lines (Dumas, Matha, Quiet, & Vey, 1996). In the gut, destruxins decrease K^+ and Ca^{++} absorption (Ruiz-Sanchez et al., 2010), induce formation of vesicles on microvilli (Dumas, Ravallec, Matha, & Vey, 1996), and increase the frequency of spontaneous contractions; the latter apparently requires the presence of Ca^{++} ions because the

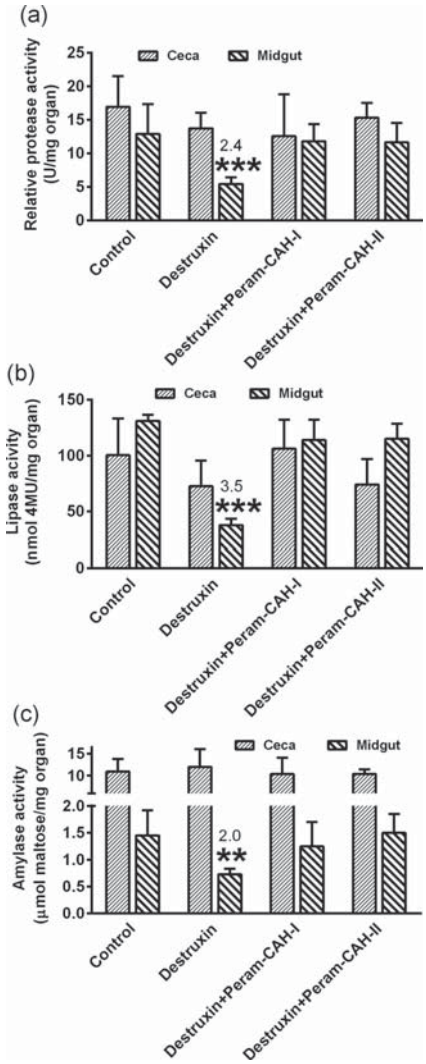

FIGURE 6 Levels of protease (a), lipase (b), and amylase (c) activities in ceca and midgut of *Periplaneta americana* adults after 24 hr incubation in medium (500 μl) in the presence of destruxin A (50 nmol in 2 μl), Peram-CAH-I (40 pmol in 2 μl) and Peram-CAH-II (40 pmol in 2 μl); controls were treated with Ringer saline only. The numbers above the columns represent fold difference of destruxin as compared with the corresponding control (midgut). Statistically significant differences between the experimental and control groups evaluated using one-way analysis of variance with Dunnett's post hoc test at the 0.1% and 1% levels are indicated by *** and **, respectively (ceca); no significant differences were found within relevant groups of midguts (midgut; $n = 5–7$)

effect is known to disappear in Ca^{++}-free medium or in the presence of Ca^{++} channel blockers (Ruiz-Sanchez et al., 2010). Ultrastructural degenerative changes following destruxin application were observed in cells of insect salivary glands (Sowjanya & Padmaja, 2008). In this study, destruxin was found to reduce enzyme activity in the midgut but this effect was eliminated by AKH application. Interestingly, destruxin A did not reduce enzymatic

activity in the ceca; however, it prevented the stimulatory effect of AKH in this organ that has been recorded in previous studies (Bodláková et al., 2017, 2018). Thus, the interaction of destruxin and AKH in the cockroach midgut appears to be complex. Our in vitro results clearly show a direct action of both agents on the gut; however, the details have not yet been elucidated. Nevertheless, one can speculate that Ca^{++} might play a role, as modulation of calcium channels is involved in the mode of action of both destruxin (Ruiz-Sanchez et al., 2010) and AKHs (Gäde & Auerswald, 2003).

In summary, the midgut showed greater sensitivity to the tested toxins than the ceca, and the role of AKHs as antistress hormones was apparent only against the action of destruxin, but not against *Isaria* infection. Future studies are needed to further explain these observations.

Overall, this study demonstrated that all three tested toxins/pathogens elicited a stress response when applied into the cockroach body as illustrated by an increased AKH level in the CNS. Their effect on the digestive processes in the cockroach gut was variable as well as the ability of AKH to counteract these effects. The venom from *H. hebetor* showed no effect on digestive enzyme activities. Infection by the fungus *I. fumosorosea* caused decreased activity of all tested enzymes in the midgut, but only decreased the activity of amylases in the ceca; application of AKHs did not reverse this inhibition. Destruxin A inhibited activity of all enzymes in the midgut but none in the ceca, and application of AKHs could reverse this inhibition. The mechanism of action underlying these observations is unknown, but our in vitro results suggest a direct action of the tested agents without mediation by other tissues or organs.

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CONFLICT OF INTERESTS

The authors declare that there are no conflict of interests.

AUTHOR CONTRIBUTIONS

D. K. conceived this research, designed experiments, interpreted the data and wrote the manuscript; N. K. and U. K. G. performed experiments and analysis. All authors read and approved the final manuscript.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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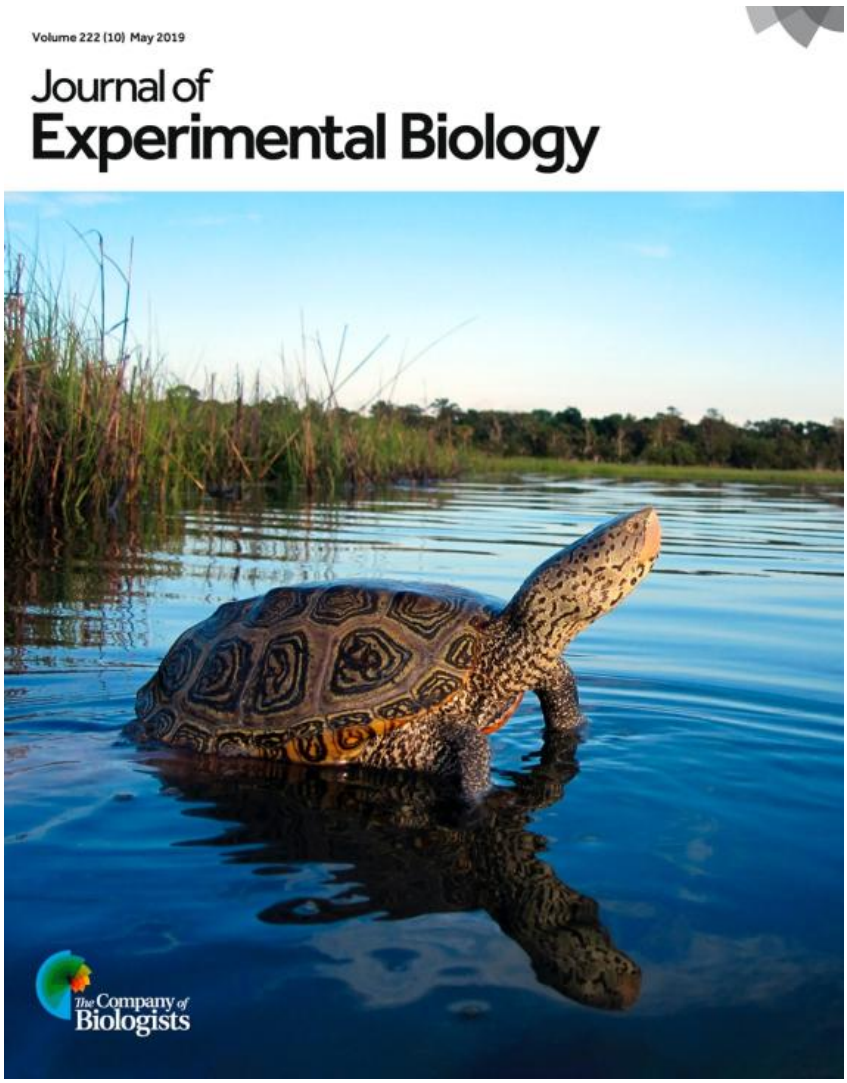
5.3. Paper III

Kodrík D., Ibrahim E., **Gautam U.K.,** Čapková Frydrychová R., Bednářová A., Krištůfek V., Jedlička P. (2019) Changes in vitellogenin expression caused by nematodal and fungal infections in insects. ***J. Exp. Biol***. 222: article no. 202853.



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RESEARCH ARTICLE

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

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ABSTRACT

This study examined the expression and role of vitellogenin (Vg) in the body of the firebug *Pyrrhocoris apterus* (Heteroptera, Insecta) during infection elicited by two entomopathogenic organisms, the nematode *Steinernema carpocapsae* and the fungus *Isaria fumosorosea*. Infection by *S. carpocapsae* significantly upregulated *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 opposite tendency was noted. 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 a level that is still sufficient for defense, but is 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 at 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 probably 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: Yolk protein, Immunity, Antibacterial activity, Antifungal activity, Entomopathogenic nematode, Entomopathogenic fungus

INTRODUCTION

Vitellogenins (Vgs), glycolipophosphoproteins mostly known and well-characterized as precursors of yolk proteins, are involved in reproduction in the majority of oviparous animals. In insects, Vgs are typically synthesized in the fat body, from where they are transported via the 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 a total molecular mass ranging from 150 to 650 kDa. Vg production is hormonally controlled. It has been 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). Additionally, Vg production is controlled by 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 reproduction but also in other aspects of insect biology, such as the 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 honeybee 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 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 upregulated 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 transgenerational 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 the anti-oxidative effect of Vg might be a crucial mechanism that extends the life span of long-lived honey bee winter workers and queens, in which Vg is synthesized in high

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levels. However, the exact mechanisms of the anti-oxidative effects of Vg and hormones in the insect body remain unclear.

In the present study, we sought to examine the role of Vg 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 infection with entomopathogenic nematode (EPN) and entomopathogenic fungus (EPF); (2) to elucidate the role of Vg in the insect body infected by EPN; and (3) to determine whether the Vg-mediated defense reaction 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.

MATERIALS 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 a constant temperature of 26±1°C under long-day conditions (18 h: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 insect treatment

Steinernema carpocapsae nematodes, originating from St Petersburg, Russia (strain NCR), were obtained 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 began.

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

Entomopathogenic fungus *Isaria fumosorosea* and insect treatment

The fungus *I. fumosorosea* isolate originating from the horse chestnut leaf miner, *Cameraria ohridella* (Lepidoptera, Gracillariidae) was obtained courtesy of Dr A. Bohatá (Agricultural Faculty, South Bohemian University, České Budějovice). The strain is deposited under number CCM 8367 as a patent culture in the Czech Collection of Microorganisms in Brno (WO2010006563A1). The spore suspension was prepared by scraping 14 day old conidiospores into a sterile solution of 0.05% (v/v) Tween®80 (Sigma-Aldrich). The suspension was filtered through sterile gauze to separate the mycelium and clusters of spores. The number of spores in the uniform suspension was counted with a Neubauer improved chamber and subsequently the suspension was adjusted to a concentration of 1×10^7 spores ml^{-1}. A 5 ml sample of the conidial suspension was added to 100 ml potato dextrose broth in a 250 ml Erlenmeyer flask, which was then placed on a shaker and incubated at 25°C and 200 rpm under constant light. After 4 days, the blastospores were harvested and injected into 7 day old males and 1 day old females at a dose of 30,000 blastospores per bug; controls were injected with Ringer saline only. The firebugs were transferred into glass jars and kept under the same conditions as for the stock culture. Hemolymph of the surviving individuals was collected 1–3 days after infection and stored at −20°C until used.

RNA and cDNA preparation

Fat body preparation

Pyrrhocoris apterus males treated with *S. carpocapsae* or *I. fumosorosea* were collected and stored at −80°C prior to processing. To monitor the 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 RNase-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, Life Technologies) with 2 µg of the corresponding total RNA and random hexamers.

Quantification of *Vg* gene expression

Quantitative real-time PCR (qPCR) was performed to evaluate *Vg* transcript levels in the fat bodies of the experimental firebugs. For these studies, the same experimental design (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 was 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 *Vg* transcript levels were determined using the threshold cycle and normalized to expression of *Rp49* (*Ribosomal protein 49*). Primers used for qPCR were: Vg (forward) CCCGACAAGTCCACAGTTATT, Vg (reverse) GCGCATTCTGTTCATGTAAGC, Rp49 (forward) CCGATATGTAAAACTGAGAAAC, and Rp49 (reverse) GGAGCATGTGCCTGGTCTTTT.

Gel electrophoresis and Vg quantification

SDS-PAGE under denaturing conditions using commercial gels (Bio-Rad, 5–20%) was performed according to Laemmli (1970) as modified by Socha et al. (1991). Typically, hemolymph samples were diluted 10-fold and 25-fold in sample buffer for male and female samples, respectively, and 10 µl was used for the analysis. The proteins separated on gels were stained with Coomassie Brilliant Blue R-250, and Vg bands were determined according to

molecular weight (MW) standards (10–250 kDa, Thermo Fisher Scientific) and reaction with specific antibody (see below); Vg band density was evaluated using a GS-800 Calibrated Densitometer with Quantity One (v4.6) 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, followed by secondary antibody Goat/HRP (1:1000 v/v; goat anti-rabbit antibody labelled with horse radish peroxidase; Sigma-Aldrich). For visualization, the Novex® ECL HRP chemiluminescent substrate reagent kit (Invitrogen) with 1:1 v/v A and B solutions was used. The developed color was documented using Intelligent Dark Box (LAS 3000, Fujifilm).

Vg isolation and antimicrobial activity

Crude Vg was isolated from the hemolymph of 3–4 day old *P. apterus* females to test its antimicrobial activity. The hemolymph samples were separated by SDS-PAGE using 10% gel according to Laemmli (1970), as described above. The gel was then 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 using 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 a 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 into micrograms of protein. Thereafter, the samples were stored at −20°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 a 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. The next day, the density of the bacterial suspension was adjusted to be 0.8 McF (McFarland bacterial density), and 0.2 ml was swabbed onto the agar plates. Vg (about 40 µg) was applied onto a sterile paper disc (Sigma-Aldrich) dried in a laminar airflow cabinet, and placed on the bacterial lawns. Gel extract (without Vg – see above) and diluting buffer (Ringer saline) were applied in the same way, as controls. The plates were incubated at 30°C overnight, and zones of growth inhibition around the paper discs were measured and their area calculated.

Mortality test

A 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 firebug males and females. Briefly, we used 7 day old males and 4 day old females, and each of the tested individuals was infected by injection of 10 nematodes in 2 µl autoclaved water into the hemocoel; controls were injected with Ringer saline only. To determine mortality, five groups

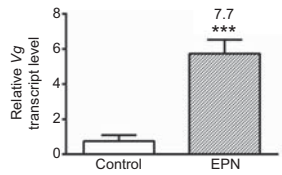

Fig. 1. Effect of *Steinernema carpocapsae* infection on vitellogenin transcript expression. Relative vitellogenin (*Vg*) transcript level (mean±s.d.) in the fat body of 8 day old *Pyrrhocoris apterus* males 1 day after *Steinernema carpocapsae* (EPN) or control treatment. ***Statistically significant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

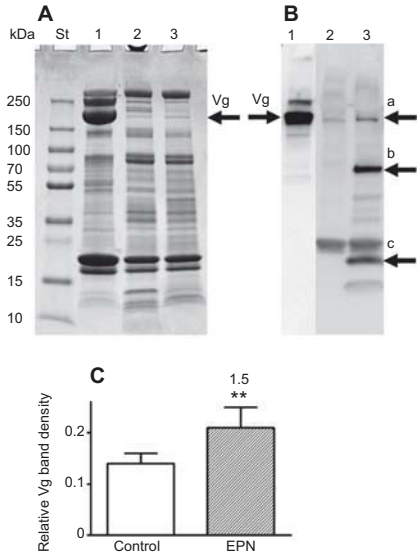

Fig. 2. Effect of *S. carpocapsae* infection on vitellogenin protein expression. (A) SDS-PAGE of hemolymph proteins of *P. apterus*: St, MW standards; 1, control 4 day old females (for Vg identification); 2, 8 day old males 1 day after control treatment; 3, 8 day old males 1 day after *S. carpocapsae* treatment. Vg bands are indicated by the arrow. (B) Western blotting of hemolymph proteins of *P. apterus*: lanes 1–3 as in A. Arrows indicate bands showing a positive reaction to anti-Vg antibody; estimated MW: a, 180 kDa; b, 65 kDa; and c, 24 kDa. (C) Relative density (mean±s.d.) of Vg bands (optical density×area, quantified from corresponding gels that are not shown) in the hemolymph of 8 day old *P. apterus* males 1 day after *S. carpocapsae* (EPN) or control treatment. **Statistically significant difference between infected and control groups at the 1% level evaluated by Student's *t*-test (n=6–7). The number above the bar represents the fold-difference of the relative Vg quantity between the EPN and control group.

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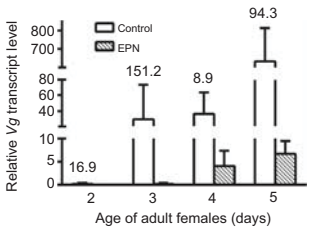

Fig. 3. *Vg* transcript expression in females following *S. carpocapsae* infection. Relative *Vg* transcript level (mean±s.d.) in the fat body of 2, 3, 4 and 5 day old *P. apterus* females 1 day after *S. carpocapsae* (EPN) and control treatment. Two-way ANOVA showed a statistically significant difference at the 0.1% level between the EPN groups and controls (n=3). Numbers above the bars represent fold-differences of *Vg* transcript levels between the EPN and corresponding control group.

(each consisting of 20 firebugs) for each experimental treatment, as well as for controls, were inspected 24 h post-treatment.

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

Data presentation and statistical analysis

The results were plotted using the graphic software Prism (Graph Pad Software, v6.0, San Diego, CA, USA). The bar graphs represent means±s.d.; the number of replicates (n) is depicted in the figure legends. Statistical differences were evaluated by Student's t-test, two-way ANOVA and one-way ANOVA using Prism software as indicated in the figure legends.

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, 1 day post-infection (Fig. 1). Vg protein level in hemolymph was also increased 1.5-fold, as visualized by SDS-PAGE (Fig. 2A,C). These observations were verified using western blotting with anti-Vg antibody (Fig. 2B). Interestingly, the anti-Vg antibody used for western blots 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 1 day after infection (Fig. 3); the extent of the inhibition ranged from about 9-fold (in 4 day old females) 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): a maximal 12-fold change (in 3 day old females) was recorded (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.

Steinernema carpocapsae elicited mortality in the treated firebugs (Fig. 5A). The mortality rate was about 2.9 times lower in females than in males, 1 day after treatment. In corresponding controls, no mortality was recorded (data not shown), perhaps as a

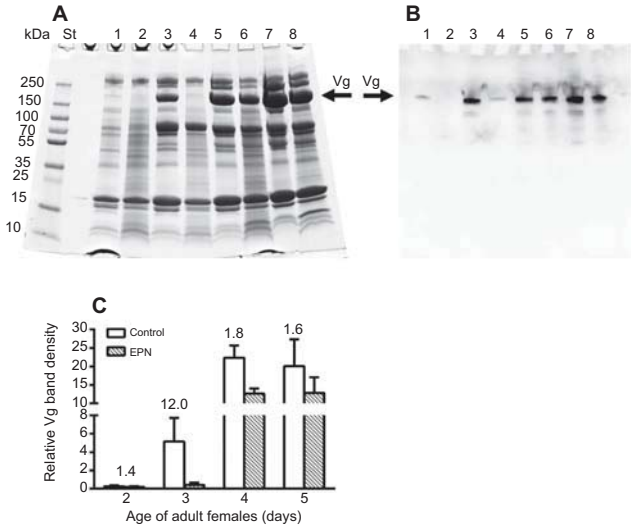

Fig. 4. Vg protein expression in females following *S. carpocapsae* infection. (A) SDS-PAGE of hemolymph proteins of *P. apterus* females: St, MW standards; 1 and 2, control (1) and *S. carpocapsae*-treated (2) 2 day old females; 3 and 4, control (3) and *S. carpocapsae*-treated (4) 3 day old females; 5 and 6, control (5) and *S. carpocapsae*-treated (6) 4 day old females; 7 and 8, control (7) and *S. carpocapsae*-treated (8) 5 day old females. In each case, measurements were obtained 1 day after *S. carpocapsae* or control treatment. Vg bands are indicated by the arrow. (B) Western blotting of hemolymph proteins of *P. apterus* females: lanes 1–8 as in A. Arrow indicates bands showing a positive reaction to anti-Vg antibody. (C) Relative density (mean±s.d.) of Vg bands (optical density×area, quantified from corresponding gels that are not shown) in hemolymph of 2–5 day old *P. apterus* females 1 day after *S. carpocapsae* (EPN) or control treatment. Two-way ANOVA showed a statistically significant difference at the 0.1% level between the EPN groups and controls (n=6–8). The numbers above the bars represent the fold-difference of the relative Vg quantity between the EPN and corresponding control group.

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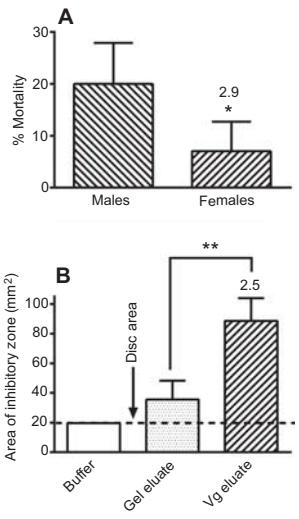

Fig. 5. *Steinernema carpocapsae*-induced mortality and Vg antimicrobial effect. (A) Effect of *S. carpocapsae* on mortality of *P. apterus* males (7 days old) and females (4 days old), 1 day after treatment (means±s.d.); there was no mortality in controls. (B) Inhibitory effect of Vg on the growth of *Xenorhabdus* spp. bacteria tested by the disc diffusion method (means±s.d.). Results evaluated 1 day after Vg application are expressed as the area of the inhibitory zone (for details, see Materials and Methods). Asterisks indicate statistically significant differences between the relevant groups at the 5% level (*A, n=5 groups with 20 adults per each) and 1% level (**B, n=5) evaluated by Student's t-test. The numbers above the bars represent the fold-difference between the relevant groups.

result of the 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.

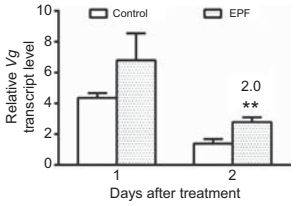

Fig. 6. Effect of *Isaria fumosorosea* infection on *Vg* transcript expression in males. Relative *Vg* transcript level (mean±s.d.) in the fat body of 8 and 9 day old male *P. apterus*, 1 and 2 days, respectively, after *I. fumosorosea* (EPF) or control treatment. **Statistically significant difference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

Isaria fumosorosea infection

Because of the slower development of the EPF *I. fumosorosea* in the fire bug body, Vg levels were monitored daily for 2–3 days after infection in the tested individuals. In the male fat body, *Vg* transcript level nominally increased 1.6-fold the day after infection, although this change did not reach the level of statistical significance (Fig. 6). However, on day 2 after 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 SDS-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 were recorded on both day 1 and day 2 after infection (Fig. 8). Similar significant changes in Vg protein level were detected in the hemolymph during the whole monitored period according to SDS-PAGE analysis (Fig. 9A,C). Identical results were also obtained using immunoblotting (Fig. 9B). Surprisingly, no differences between male and female firebugs were recorded when mortality was monitored for 1–3 days after treatment with *I. fumosorosea* (Fig. 10).

DISCUSSION

Pathogenic organisms elicit severe stress in the host body, which results in the disruption of functional homeostasis (Ivanović and Jankovic-Hladni, 1991) and activation of characteristic defense response to eliminate or at least 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 also includes Vgs, whose involvement in insect defense systems 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 in 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 the hemocoel. Once the nematodes are inside the insect body, the infection develops quite quickly; therefore, we monitored the effect of the EPN at just 1 day post-infection: 1 day later, mortality reached almost 100% (data not shown). During development in the host body, EPNs produce various venoms and toxins, which are generated by the 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 the EPNs against the defense system of their insect host, and afterwards, they kill the host and use its organs as a source of nutrients for growth and development. The insects protect themselves by clotting cascades, and 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 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 Vg protein level in the hemolymph. To the best of our knowledge, this is the first report of a stimulatory effect of the

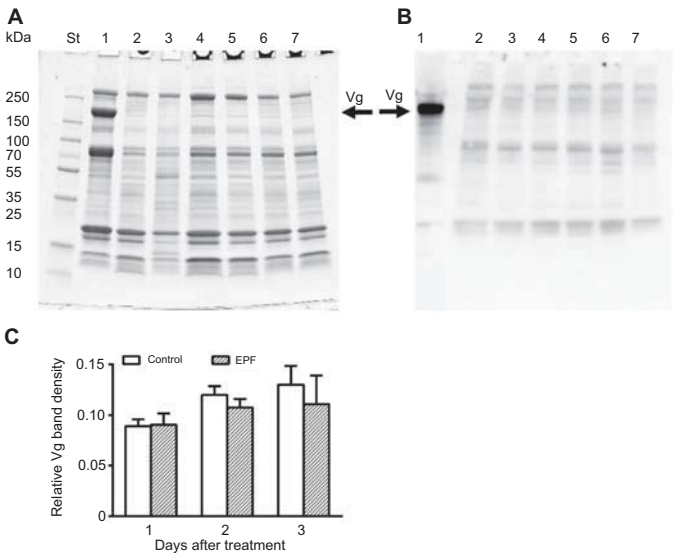

Fig. 7. Effect of *I. fumosorosea* infection on Vg protein expression in males. (A) SDS-PAGE of hemolymph proteins of *P. apterus*: St, MW standards; 1, control 4 day old females (for Vg identification); 2 and 3, control (2) and *I. fumosorosea*-infected (3) 8 day old males 1 day after treatment; 4 and 5, control (4) and *I. fumosorosea*-treated (5) 9 day old males 2 days after treatment; 6 and 7, control (6) and *I. fumosorosea*-treated (7) 10 day old males 3 days after treatment. Vg bands are indicated by the arrow. (B) Western blotting of hemolymph proteins of *P. apterus*: lanes 1–7 as in A. Arrow indicates bands showing a positive reaction to anti-Vg-antibody. (C) Relative density (mean±s.d.) of Vg bands (optical density×area, quantified from corresponding gels that are not shown) in hemolymph of 8, 9 and 10 day old *P. apterus* males, 1, 2 and 3 days, respectively, after *I. fumosorosea* (EPF) or control treatment. No statistically significant difference between infected and control groups evaluated by two-way ANOVA at 5% was recorded (n=6–8).

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 the combined effect of the exposure to both. However, it is known that both organisms are insect pathogens (Herbert and Goodrich-Blair, 2007; Waterfield et al., 2009). Further, it seems 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.

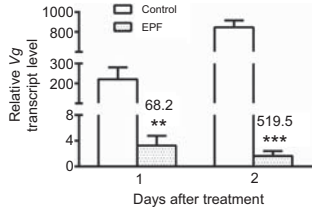

Fig. 8. Effect of *I. fumosorosea* infection on *Vg* transcript expression in females. Relative *Vg* transcript level (mean±s.d.) in the fat body of 2 and 3 day old *P. apterus* females, 1 and 2 days, respectively, after *I. fumosorosea* (EPF) or control treatment. Asterisks indicate statistically significant differences between infected and control females at the 1% level (**) and 0.1% level (***) evaluated by Student's *t*-test (n=3). The numbers above the bars represent the fold-difference of *Vg* transcript levels between the EPF group and control.

Vg plays a key role as an irreplaceable component of yolk in eggs developing in the female body; however, the (perhaps secondary) role of Vg in immunity seems to be important as well. Evidence for the presence of Vg in the male body has been rather scarce, but Vgs have been identified in *P. apterus* (Němec et al., 1993), *Apis mellifera* (Villar and Grozinger, 2017) and *Bombus terrestris* (Jedlička et al., 2016) males. Nevertheless, a comprehensive understanding of the role of Vg in insect males is missing.

Furthermore, the multi-faceted role of Vg in the 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*, this finding is easily explained: one can speculate that a 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 stressful 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 during increased energy 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 at the end of the female reproductive cycle (Moshitzky and Applebaum,

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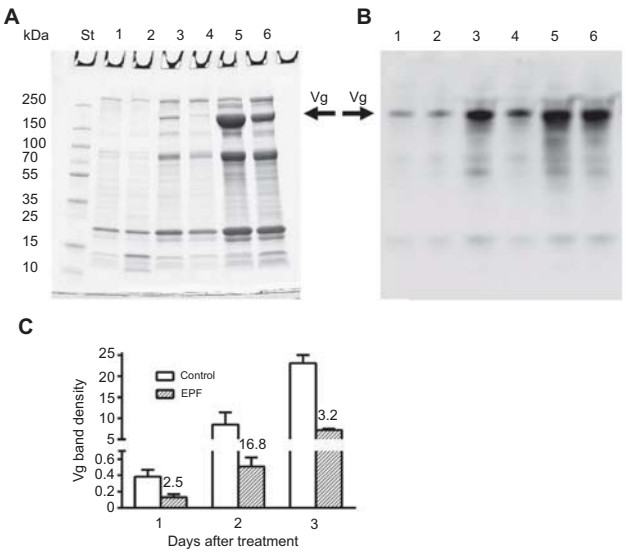

Fig. 9. Effect of *I. fumosorosea* infection on Vg protein expression in females. (A) SDS-PAGE of hemolymph proteins of *P. apterus* females: St, MW standards; 1 and 2, control (1) and *I. fumosorosea*-infected (2) 2 day old females 1 day after treatment; 3 and 4, control (3) and *I. fumosorosea*-infected (4) 3 day old females 2 days after treatment; 5 and 6, control (5) and *I. fumosorosea*-infected (6) 4 day old females 3 days after treatment. Vg bands are indicated by the arrow. (B) Western blotting of hemolymph proteins of *P. apterus* females: lanes 1–6 as in A. Arrow indicates bands showing a positive reaction to anti-Vg-antibody. (C) Relative density (mean± s.d.) of Vg bands (optical density×area, quantified from corresponding gels that are not shown) in hemolymph of 2, 3 and 4 day old *P. apterus* females, 1, 2 and 3 days, respectively, after *I. fumosorosea* (EPF) or control treatment. Two-way ANOVA test showed a statistically significant difference at the 0.1% level between the EPN groups and controls (n=6–10). The numbers above the bars represent the fold-difference of the relative Vg quantity between the EPN and corresponding control group.

1990). This process is independent of nutrient mobilization because Vg inhibition occurs at hormone titers about one-tenth those necessary for nutrient mobilization from the fat body: thus, the two activities stimulated by adipokinetic hormone are not overlapping (Carlisle and Loughton, 1986). The mechanism of Vg function 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 probably kills entomotoxic bacteria. It has not been established whether Vg affects EPNs; therefore, we cannot definitely exclude this. An antibacterial effect of Vg has already been described in several studies. Singh et al. (2013) showed that Vg of the silkworm *Bombyx mori* has 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 the bacterial surface, inducing structural damage in the cell wall, which resulted in membrane disruption and permeabilization. All these data suggest that Vg is an antibacterial agent with a wide spectrum of action.

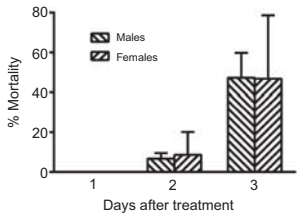

Fig. 10. *Isaria fumosorosea*-induced mortality. Effect of *I. fumosorosea* on mortality of *P. apterus* males (7 days old) and females (4 days old), 1–3 days after infection (means±s.d.); there was no mortality in controls. No statistically significant difference between males and females evaluated by two-way ANOVA at the 5% level was recorded (n=11 groups with 20 adults per each).

EPFs, such as *I. fumosorosea* used in this study, usually start their infection by breaking the host cuticle and physically penetrating the host body, using various enzymes, such lipases, proteases, chitosanases and chitinases (Hajek and Leger, 1994; Ali et al., 2010), 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 these effective mechanisms, the whole process of EPF infection is rather slow – to speed it up, we used injection of blastospores, in 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 the EPF effect for 2–3 days after injection. The response of the *P. apterus* male body to EPF infection differed from that to EPN infection. The first significant upregulation of *Vg* transcription in the fat body was observed 2 days post-infection; however, surprisingly, *Vg* mRNA levels in the hemolymph did not show the same trend, as a 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 in responses to infections with EPN and EPF. In contrast, the response of firebug females to EPF infection was quite similar to that elicited by EPN: both mRNA and protein Vg levels were significantly down-regulated. A 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 were discussed above. In addition, an active involvement of Vg in the defense reaction of *A. mellifera* against the entomopathogenic fungus *B. bassiana* was recently described by Park et al. (2018), who demonstrated that Vg behaves as a typical anti-microbial peptide. *Apis mellifera* Vg bound to *B. bassiana* 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 an 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. Clearly, Vg proteins play an important role in the defense against various types of infection, including those caused by EPN and EPF. Furthermore, Vg transcription and protein synthesis are modulated depending on the type of infection and the sex of the infected insect. Vg acts directly against *Xenorhabdus* spp. bacteria isolated from the entomopathogenic complex of *S. carpocapsae*. Other mechanisms of Vg 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 a different mortality response (in both sexes) to the two studied pathogens.

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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., R.Č.F., A.B.; Investigation: D.K., R.Č.F., A.B.; Writing - original draft: D.K.; Writing - review & editing: D.K., R.Č.F.; Supervision: D.K.; Project administration: D.K.; Funding acquisition: D.K.

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Supplementary information
Supplementary information available online at
http://jeb.biologists.org/lookup/doi/10.1242/jeb.202853.supplemental

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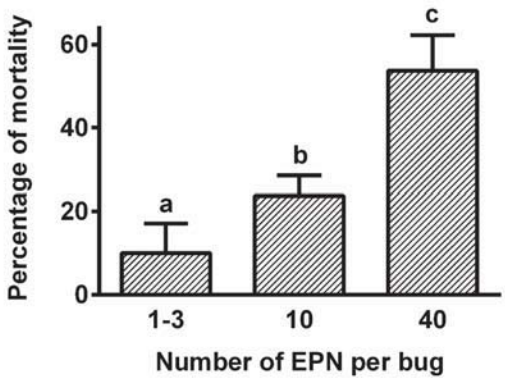

Fig. S1. The effect of various doses of *S. carpocapsae* (EPN) on mortality of *P. apterus* males (8-day old) 1 day after the treatment; mortality in controls is not shown being negligible. Statistically significant difference among infected groups at 5% level evaluated by one-way ANOVA with Tukey's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

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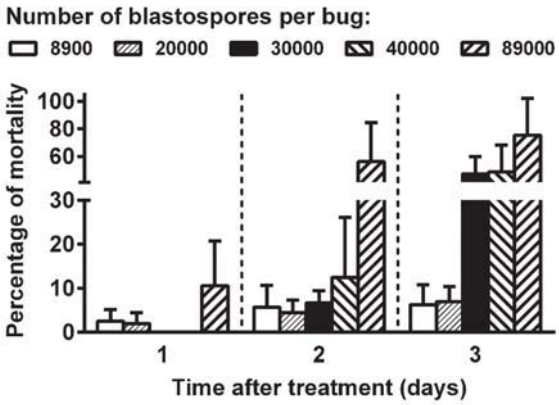

2-way ANOVA

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).

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6.1. List of abbreviations

AKH – Adipokinetic hormone

AKHR – AKH receptor

CA – Corpora allata

CAT – Catalase

CC – Corpora cardiaca

CNS – Central nervous system

DOPA – Dihydroxyphenyl alanine

Ec – Ecdysteroids;

EPF– Entomopathogenic fungi

EPN– Entomopathogenic nematode

GPCR – G protein coupled receptor

GSH – Reduced glutathione

GST – Glutathione S-transferase

HTH – Hypertrehalosemic hormone

JH – Juvenile hormone

LPS – Lipopolysaccharide

OS – Oxidative stress

PKC– Protein kinase C

ProPO – Prophenoloxidase

ROS – Reactive oxygen species

SOD – Superoxide dismutase

Vg – Vitellogenin

6.2. Curriculum vitae

Personal data

Full Name:	Umesh Kumar Gautam
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Research/Work Experience

Aug 2015 to until now

Research assistant (Part time), Institute of Entomology, Czech Academy of Science, Ceske Budejovice, Czech Republic

Research project- **Effect of the entomopathogenic fungus *Isaria fumosorosea* on physiological processes in insects**

Oct 2018 to July 2019

TÜBİTAK – 2216 Research fellow, Department of Plant Protection, Faculty of Agriculture, Süleyman Demirel University, Isparta, Turkey

Research project - **Functional study of adipokinetic hormone and its role in infections elicited by entomopathogenic fungi**

Sept 2014- July 2015

International Training Course Fellow, Institute of Genetic, Hungarian Academy of Sciences, Szeged, Hungary

Research project - **Nuclear localization of an actin-binding protein in *Drosophila melanogaster***

July 2013 – June 2014

Master of Science in Biochemistry, Department of Biochemistry University of Hyderabad, Hyderabad, India

Thesis Project **- Protective Effect of *Leucas aspera* Against Bromobenzene Induced Hepatotoxicity in Rats**

Educational profile

Aug 2015 to until now	**PhD. Student,** University of South Bohemia in Ceske Budejovice, Czech Republic
Course studied	Physiology, Immunology, Cell biology and Developmental biology
Aug 2014- July 2015	**International Training Course Fellow**, Institute of Genetic, Biological Research Centre, Szeged, Hungary
Course studied	Lectures and practical demonstrations on Contemporary Experimental biology and Developmental biology Research Practice
July 2012 – June 2014	**Master of Science in Biochemistry**, Department of Biochemistry, University of Hyderabad, Hyderabad, India.
Course studied	1-Intermediary Metabolism, 2-Molecular Biology, 3-Basic Bioinformatics, 4- Basic Immunology, 5- Bioenergetics, 6- Genetics,7-Epigenetics, 8- Clinical Biochemistry, 9-Biodiversity, 10-Enzymology, 11-Structral Biology,12- Endocrine biochemistry 13-Molecular Neuroscience.

6.3. Publications

1- **Gautam U.K.,** Bohatá A., Shaik HA., Zemek R., Kodrík D. (2020) Adipokinetic hormone promotes infection with entomopathogenic fungus *Isaria fumosorosea* in the cockroach *Periplaneta americana*. ***Comp Biochem Phys C***. 229: 108677.

2- Karbusová N., **Gautam U.K.**, Kodrík D. (2019) Effect of natural toxins and adipokinetic hormones on the activity of digestive enzymes in the midgut of the cockroach *Periplaneta americana*. **Arch. *Insect Biochem. Physiol***. 101: e21586.

3- Kodrík D., Ibrahim E., **Gautam U.K.**, Čapková Frydrychová R., Bednářová A., Krištůfek V., Jedlička P. (2019). Changes in vitellogenin expression caused by nematodal and fungal infections in insects. ***J. Exp. Biol***. 222: article no. 202853.

Advanced Techniques & Experiences

- Both team and independent laboratory skills and ability to study new impulses and information
- Molecular biology techniques including DNA isolation, agarose gel electrophoresis, bacterial transformation, transfection, DNA cloning and gene expression analysis
- Biochemical methods including oxidative markers measurement, scavenging assays, protein electrophoresis, ELISA, Western blotting, HPLC techniques and spectrophotometer assays
- Handling animal (rats) and insects (silkworms, firebugs, fruit flies, cockroaches)
- Experiences with cell culture techniques and imaging analysis of Drosophila Cell line SR2+

- R and prism GraphPad
- **Teaching experience**-Teaching assistant in practical course: Developmental Biology and Animal Physiology

6.4. Conferences and workshops attended

Kayseri, Turkey: Oral presentation in 7th International Entomopathogens and Microbial Control Congress- Enhancement of efficacy the entomopathogenic fungus, *Beauveria* spp. on the aphids, *Myzus persicae* by adipokinetic hormone (11-13 Sept 2019)

Adana, Turkey: oral presentation in 1st International Molecular Plant Protection Congress- Entomopathogenic fungus as potential biocontrol agent modulating production of vitellogenin protein in the firebug *Pyrrhocoris apterus* (10-13 April 2019)

Košice, Slovakia: oral presentation in 93rd Physiological Days -Insect model species and their mutants used for adipokinetic hormone studies (31Jan to 02 Feb)

Szeged Hungary: oral presentation in Straub Day Biological Research Centre -Unraveling the function of cytoskeletal proteins in the nucleus (03-04 June 2015)

Hyderabad, India: Participated in the "82nd Annual Meeting of The Society of Biological Chemists (India) and International conference on "Genomes: Mechanism and Function" (02-05 Dec 2013)

Hyderabad, India: Attended International workshop on

Frontiers in Biological Scienc**e** (08-09 April 2013)

Bangalore, India: Attended Pre-Interview Familiarisation Program at Indian Institute of Science Bangalore (21-31 May 2011)

Academic Distinctions

- **TÜBİTAK – 2216 Research fellow,** Department of Plant Protection, Faculty of Agriculture, Süleyman Demirel University, Isparta, Turkey (Oct 2018 to July 2019).
- **Hungarian Fellowship for International Students**: Fellowship for International Training course from Hungarian Academy of Sciences at the Biological Research Center Szeged, Hungary (Sept 2014 to July 2015).
- **Two years research and study scholarship:** Award for Boarding/Boarding-cum Lodging Allowance & Contingency scholarship master's degree category during M.SC studies at Department of Biochemistry, School of Life Science, University of Hyderabad, India (Sessions July 2012 to May 2014).

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