School of Doctoral Studies in Biological Sciences
University of South Bohemia in České Budějovice
Faculty of Science

Study of adipokinetic hormone role in insects stressed by entomopathogenic nematodes

Ph.D. Thesis

RNDr. Ing. Emad Ibrahim

Supervisor: Prof. RNDr. Dalibor Kodrík, CSc.

Faculty of Science, University of South Bohemia in České Budějovice
Institute of Entomology, Biology Centre CAS České Budějovice

České Budějovice 2019

This thesis should be cited as:
Ibrahim E. (2019): Study of adipokinetic hormone role in insects stressed by entomopathogenic nematodes. Ph.D. Thesis, University of South Bohemia, Faculty of Science, School of Doctoral Studies in Biological Sciences, České Budějovice, Czech Republic, 91 pp.

Annotation

In this thesis, the effect of infection elicited by entomopathogenic nematodes (EPNs) *Steinernema carpocapsae* on *Pyrrhocoris apterus* and *Drosophila melanogaster* models were evaluated, and a role of adipokinetic hormones (AKHs) during the infection was characterized. These were monitored by determination of mortality, and various biochemical and physiological characteristics such as AKH levels both in the central nervous system (CNS) and in hemolymph, AKH gene expression in CNS, level of anti-oxidative stress markers, general metabolism and level of nutrients in normal and genetically modified insects. At *P. apterus* the mortality tests revealed that application of AKH increases the efficacy of EPN treatment. This result was confirmed using the firebugs with AKH receptor deficiency. Further, the increase of AKH expression and AKH levels in CNS and hemolymph seemed to be coordinated after the nematode treatment. At the *D. melanogaster* model also, the effect of adenosine into the above-mentioned characteristics was included. For this, mutants in AKH (AHK^1), adenosine receptor ($AdoR^1$) genes, and in both these genes together (AHK^1 $AdoR^1$ double mutant) were employed. Altogether, the results confirmed the involvement of AKH, and partially also adenosine into the antistress defence reactions elicited by the nematobacterial infection. Finally, the last part of the study was focused on examination of the vitellogenin (Vg) role in the defence reaction in firebug body *P. apterus* affected by two entomopathogenic organisms, the nematode *S. carpocapsae* and the fungus *Isaria fumosorosea*. The results revealed that Vg proteins play an important role in the defence against both types of the infections and are also able to kill entomopathogenic bacteria *Xenorhabdus nematophila*, that are symbionts of *S. carpocapsae* and that increase toxicity of this nematode.

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.

České Budějovice, 25.03.2019 Emad Ibrahim

...…….

This thesis originated from a partnership of Faculty of Science, University of South Bohemia, and Institute of Entomology, Biology Centre of the ASCR, supporting doctoral studies in the Immunology and Developmental Biology study program.

Přírodovědecká fakulta
Faculty of Science

Jihočeská univerzita v Českých Budějovicích
University of South Bohemia in České Budějovice

BIOLOGY CENTRE CAS

Entomologický ústav
Institute of Entomology

Financial support:

This thesis was supported by the grant Czech Science Foundation 17-03253S

Acknowledgement

The research in this thesis would have taken far longer to complete without the encouragement of many others. It is a delight to acknowledge those who have supported me over the last four years.

Firstly, I would like to express my deep gratitude to my supervisor and mentor Prof. Dalibor Kodrík, for the continuous support of my PhD study, for his patience, motivation, encouragement and immense knowledge. His guidance helped me in all the time of research and writing of this thesis. I have been extremely lucky to have a supervisor who cared so much about my work, and who responded to my questions and queries so promptly.

I would also like to express my gratitude to Dr. Pavel Hyršl and Dr. Pavel Dobeš, for the co-operation, useful discussion and comments that they suggested me widen my knowledge in entomopathogenic nematodes field throughout the course of my study.

My sincere thanks also go to Prof. Ulrich Theopold, who provided me with an opportunity to join his team as an intern, and who gave access to the laboratory and research facilities. I am also grateful for having a chance to meet so many wonderful people and professionals who led me through this internship period, especially for Alexis Dziedziech and Dilan Khalili.

I am very thankful to my lab-mates, namely Helena Radová, Haq Shaik, Umesh Gautam, Tereza Koníková, Kosťa Vinokurov, Markéta Hejníková and Milada Zemanová previously, for their valuable support in the lab during my experiments, coffee and tea breaks and enjoyable atmosphere. Very special thanks go to Daniela Hlávková, for the help and advice during my PhD study, without whom the learning curve would have been very much steeper. The timeless inspiration and encouragement that she gave have put me through this journey. I also thank Andrea Bednářová, for all her support especially during my state exam.

I am indebted to all my friends, namely Abdoallah, Ketta, Talal, Yamen, Yu-Hsien, Houda, Sazzad, Asma, Sniha, Bulah, Lenka and Vasek, the days would have passed far more slowly without their support, both at České Budějovice and around the Czech Republic, whom I thank for providing such a rich source of conversation, education and entertainment.

I am also grateful to the funding received from the Egyptian mission in Cairo to undertake my PhD.

Finally, I would like to thank my parents for their love and encouragement, without whom I would never have enjoyed so many opportunities. I would like to express my very special thanks to my wife Hagar, for being a very special part of me. I am indebted to her with an extreme sense of gratitude for the love, help and support that she has been showing through the period of our togetherness. Special thanks are due for her patience and encouragement during the writing of this thesis.

List of papers and author's contribution

Paper 1

Ibrahim E., Hejníková M., Shaik H. A., Doležel D., Kodrík D. (2017) Adipokinetic hormone activities in insect body infected by entomopathogenic nematode. *J. Insect Physiol*. 98: 347-355.

Paper 2

Ibrahim E., Dobeš P., Kunc M., Hyršl P., Kodrík D. (2018) Adipokinetic hormone and adenosine interfere with nematobacterial infection and locomotion in *Drosophila melanogaster*. *J. Insect Physiol*. 107: 167-174.

Paper 3

Kodrík D., Ibrahim E., Gautam U. K., Frydrychová R., Bednářová A., Krištůfek V., and Jedlička P. Changes in vitellogenin expression caused by nematodal and fungal infections in insects. *J. Exp. Biol.* Submitted manuscript.

Co-author agreement

The corresponding author of the papers included in this thesis, hereby confirms that Emad Ibrahim contributed significantly to these publications, according to the statements above.

.....

Prof. RNDr. Dalibor Kodrík, CSc.

Contents

1. Introduction

1.1. Stress in insects

Most studies use the word "stress" to describe a reaction of an organism to a wide variety of potentially challenging environmental factors during their life such as chemical (pesticides, drugs, metals, smog, abnormal oxygen concentration, etc.), physical (radiation, temperature, noise, vibration, etc.), physiological (injury, aging, inflammation, etc.) factors, and pathogen organisms (bacteria, nematodes, parasitic arthropods, parasitoids etc.), that may upset functional homeostasis when the limits of their tolerance are exceeded (Ivanovič and Jankovič-Hladni, 1991).

These stressors elicit a characteristic response, which is often referred to as a generalized defense response. The generalized defense response exists at both the organismal and cellular levels (Hightower, 1991). Insects exposed to unfavorable environmental condition produce an acute stress response. During that, the feeding and reproduction are curtailed, heart and gas exchange rates increase, metabolic processes release stored energy, and oxygen and nutrients are directed to those sites in the body that are affected the most. In addition, insects employ a wide range of physiological and behavioral stress responses to unfavorable conditions: they include also a dormancy state, very often in the phase of egg or pupa, to avoid those conditions.

A special example of stress is oxidative stress (OS), the imbalance towards an increase in the prooxidant over the capacity of the antioxidant and might lead to potential biological damage. Intensive production of reactive oxygen species (ROS) results in oxidative damage to the cellular lipids, proteins, or DNA inhibiting their normal functions, with irreversible impact for the whole organism, leading to death. As OS results from the metabolic reactions that use oxygen can also be induced by environmental factors such as pesticides, chemicals, food sources, UV light, irradiation promote ROS (Ahmad et al., 1995; Bi and Felton, 1995). Insects have evolved several antioxidant defense mechanisms which includes both enzymatic components such as superoxide dismutase, catalase, ascorbate peroxidase and glutathione S-transferase (Ahmad et al., 1989; Felton and Summers, 1995; Krishnan et al. 2009) and non-enzymatic components such as glutathione, vitamins C and E, pyruvate, flavonoids, carotenoids, urate and many plant-derived antioxidants to reduce, destroy, or prevent the effects of ROS (Valko et al., 2007).

In mammals the humoral defense response pathways are controlled by the hypothalamic - pituitary - adrenal axis with help of a suite of corresponding hormones (Jansen et al., 1995; Smith and Vale, 2006). In insects, the defense biochemical and physiological reactions under unfavorable conditions are controlled predominantly by members of the adipokinetic hormone/red pigment-concentrating hormone family (AKH/RPCH family) (Gäde et al. 1997; Kodrík 2008).

1.2. Adipokinetic hormones

Adipokinetic hormones (AKHs) are insect neuropeptides that are synthesized, stored and released from the neurosecretory cells of the corpora cardiac (CC), a neurohemal organ connected to the brain (Gäde et al., 1997). However, in some insect species, such as *Locusta migratoria* (Schooneveld et al., 1985; Moshitzky et al., 1987a; Bray et al., 1993), *Carausius morosus* and *Sarcophaga bullata* (Clottens et al., 1989), *Aedes aegypti* (Kaufmann et al., 2009), and *P. apterus* (Kodrík et al., 2003), small quantities of AKH are also found in the brain.

Primary structures of AKHs are known for many insect species: they are small oligopeptides consisting of 8 to 10 amino acids containing at least two aromatic amino acids. All AKHs contain an N-terminus pyroglutamate residue, C-terminus amide, and possess tryptophan in position 8 and phenylalanine or tyrosine in position 4 (Gäde et al., 1997).

AKH signal transduction at the cellular level is well documented in the fat body (see Gäde and Auerswald, 2003). Because AKH as a peptidic hormone is not able to penetrate freely the cell membranes, its message is transduced via specific membrane receptors (Park et al., 2002; Staubli et al., 2002). This receptor which is structurally related to receptors of the vertebrate gonadotropin-releasing hormone is linked to a G-protein that activates corresponding biochemical pathways leading to the production of energy-rich substrates. This includes activation of the alpha subunit of G-protein (G_α) by replacing GDP by GTP, which stimulates the production of enzyme adenylate cyclase, which generates cyclic AMP that serves as a secondary messenger molecule. In some insect species, the $G_{\beta\gamma}$ subunit activates phospholipase C pathways which catalysis the hydrolysis of membrane phospholipid, phosphatidylinositol 4,5, bisphosphate, resulting in the production of second messenger diacylglycerol and inositol phosphates, including inositol 1,4,5-trisphosphate. Both pathways involving the presence of extracellular and/or intracellular Ca^{2+}, then the membrane protein kinase C is activated and consecutively triggers relevant enzyme activations (Gäde and Auerswald, 2003).

AKH receptors (AKHRs) have been cloned or deduced from genomic sequences of several insect species including the fruit fly, *D. melanogaster* (Park et al., 2002), silkworm, *Bombyx mori* (Huang et al., 2010), cockroach, *Periplaneta americana* (Hansen et al., 2006; Wicher et al., 2006), firebug, *P. apterus* (Ibrahim et al., 2017), aphid *Acyrthosiphon pisum* (Li et al., 2013), cricket, *Gryllus bimaculatus* (Konuma et al., 2012), mosquito, *Anopheles gambiae* (Belmont et al., 2006), fleshly, *Sarcophaga crassipalpis* (Bil et al., 2016), tsetse fly, *Glossina morsitans* (Caers et al., 2016), beetle, *Tribolium castaneum* (Hansen et al., 2010), the bamboo aphid, *Pseudoregma bambucicola* (Jedličková et al., 2015) and triatomine, *Rhodnius prolixus* (Alves-Bezerra et al., 2016). In the honey bee, *Apis mellifera* genome, an AKH receptor ortholog has been identified (Hauser et al., 2006,) but in several studies, it was indicated that an AKH signaling system would not be functional in *A. mellifera* because of the inefficacy identification of AKH by mass spectrometry methods (Veenstra, 2014). However recently, the AKH

signal has been documented in the glandular part of the *A. mellifera* CC (Sturm et al., 2016).

More than 60 different modifications of AKHs have been characterized at present, and their physiological effects investigated in all major insect orders (Gäde, 2009; Kodrík et al., 2015). Generally, these hormones are a typical example of neuropeptides with complex functions in the control of insect energy metabolism, including the mobilization of different kinds of energy reserves such as lipids, carbohydrates, and/or certain amino acids (Gäde et al., 1997; Gäde and Goldsworthy, 2003). Furthermore, AKHs are pleiotropic in their functions, with a lot of activities associated to their metabolic role (Kodrík, 2008) such as the stimulation of neuronal signaling (Milde et al., 1995), stimulation of general locomotion (Socha et al., 1999), and increase of muscle tonus and heart beating (O'Shea et al., 1984; Scarborough et al., 1984). These hormones are also known to regulate the starvation-induced foraging behavior in *Drosophila* (Lee and Park, 2004), enhancement of antioxidant mechanisms (Kodrík et al., 2007, Kodrík et al., 2015; Večeřa et al., 2007), enhancement of food intake and digestive processes in insect gut (Kodrík et al., 2012; Bil et al., 2014; Bodláková et al., 2017, Bodláková et al., 2018), and interact with the cellular and humoral immune system (Goldsworthy et al., 2002, Goldsworthy et al., 2003).

1.2.1. Adipokinetic hormones and their interactions with oxidative stress

It has been found that there is a feedback regulation between OS and AKH actions. The active role of AKH in protection of insects against OS was reported in a number of papers published in the last decade (Kodrík et al., 2007; Večeřa et al., 2007, 2012; Huang et al., 2012; Bednářová et al., 2013a,b, 2015) and reviewed by Krishnan and Kodrík (2012) and Kodrík et al. (2015a). The results revealed that OS increased the level of AKH in insect body and that exogenous AKH restored anti-oxidative stress reactions in insect tissues (as well as in vitro organ cultures). Those hormones are involved in the activation of protective mechanisms against the OS and can thus affect both enzymatic or non-enzymatic OS markers such as glutathione, protein carbonylation, lipid peroxidation or total antioxidant capacity (Krishnan and Kodrík, 2012; Kodrík et al., 2015a).

AKH-elevating effect has been governed by several stressors and it seems that the increase is species and/or stressor specific. For example, application of chemical stressor herbicide paraquat (1,1'-dimethyl-4,4'-bipyridylium dichloride hydrate) in the *P. apterus* for 4 hours exposure, increased the AKH level in hemolymph about 5 times, but the same treatment had no effect on the AKH level in the CNS (Večeřa et al., 2007). Also, in the Colorado potato beetles *Leptinotarsa decemlineata,* the paraquat treatment increased the AKH level about 1.9 times in CNS and 2.7 times in hemolymph (again 4 hours after the treatment). In the same species, the AKH level was increased in CC and hemolymph when the individuals were fed on genetically modified potatoes expressing Cry 3Aa-*Bacillus thuringiensis* and *Galanthus nivalis* agglutinin toxins (Kodrík et al., 2007). Similar results were obtained when hydrogen peroxide was injected in the *P. apterus* (Bednářováet al., 2013).

The OS markers involve several enzymes. Among them, superoxide dismutase (SOD) and catalase (CAT) are standing in the first line of the defense system against OS (Scott et al., 1991; Burlakovaet al., 2010). The enhancement of expression of SOD and CAT genes were increased during the OS caused by different stressors (Whiteside and Hassan, 1987; Farr and Kogoma, 1991). Further, feeding of tannic acid by *Spodoptera littoralis* larvae resulted in significant increase of CAT gene expression, however, the co-treatment with AKH reduced it back to the control level (Večeřa et al., 2012). Also, the activity of glutathione-S-transferase (GST) decreased significantly in the *S. littoralis* larvae fed on tannic acid, and that returned to the control level after the co-treatment with AKH (Večeřa et al., 2012). All these studies indicate that AKH is somehow actively involved in the control of the SOD, CAT and GST in insects.

It has also been observed that AKHs can affect non-enzymatic OS markers such as glutathione (GSH), protein carbonylation, lipid peroxidation or total antioxidant capacity. For example, in *L. decemlineata* and *P. apterus* hemolymph, the GSH level significantly increased (almost two-fold) after the AKH injection but was significantly decreased (about 2 to 3 times) after the paraquat treatment (Kodrík et al., 2007; Večeřa et al., 2007). Nevertheless, co-injection of AKH with paraquat resulted in the return of GSH content back to the control level. On the other hand, a different effect was obtained in *S. littoralis*, where the GSH level significantly reduced after tannic acid feeding, while the application of AKH elicited no significant effect in intact insects and similarly, the same application elicited no relevant effect in GSH level (Večeřa et al., 2012). However, it is quite possible that GSH alone (induced by AKH) could not be the one to confer enhanced antioxidant capacity to the hemolymph.

Another important OS marker is protein carbonylation, during the stress situation induced by chemical stressors such as paraquat, hydrogen peroxide, tannic acid application, the level of the protein carbonyl significantly elevated in insect hemolymph, but their co-injection with AKH returned the carbonyl levels back to the control level. (Kodrík et al., 2007, Večeřa et al., 2007, Bednářová et al., 2013b). In the same time, AKH injection alone (without any stressor) does not affect the carbonyl levels to those below control values. This indicates that possibly a stressor action is needed for AKH to exert its response by curbing formation of carbonyls.

The list of AKH activities in its anti-oxidative response role also involves the control of lipid peroxidation in the cell membranes (Bednářová et al., 2013a). The level of lipid peroxidation significantly increased in *P. apterus* CNS after incubation with hydrogen peroxide in vitro, while the incubation of CNS with hydrogen peroxide and AKH reduced lipid peroxidation level (comparable to control one). Interestingly, application of AKH alone reduced the lipid peroxidation level to even below control values. It suggests a clear role of AKH in inhibiting lipid peroxidation but the mechanism by which it exerts this effect is unclear (Bednářová et al., 2013a).

Hence, all these results support that AKHs comprehensively control the anti-oxidative response and suggest that there must be some other additional mechanisms by which AKH acts to enhance antioxidant response.

1.2.2. Adipokinetic hormones and their interactions with insecticides

As mentioned above AKHs participate in the control of various stress responses; they directly regulate or participate in response to stress factors. Severe stress in insect body is primarily caused by the application of insecticides. Requirements of the insects affected by them are essentially similar: insects must activate their defense reactions and mobilize energy stores to eliminate or at least to minimize the toxic impact of insecticides on physiological and other functions.

It has been found that application of several neurotoxic insecticides (permethrin, endosulfan, malathion, pirimiphos-methyl and deltamethrin) on two model insect species the firebug, *P. apterus* (Kodrík and Socha, 2005; Kodrík et al., 2010; Velki et al., 2011) and the red flour beetle, *T. castaneum* (Plavšin et al., 2015) affected the AKH level in insect body. This clearly indicates the involvement of this hormone into the activation of protective mechanisms against the toxic stress. However, the co-application of AKH and toxins, topically or by injection, surprisingly enhanced the toxin efficacy in the insect body. This result seems to be illogical at first glance, but they are documented by a set of the above-mentioned papers (reviewed by Kodrík et al., 2015b). The mechanism of this synergistic action is not known in detail; however, we have hypothesized that AKHs might intensify the toxin action by accelerating metabolite exchange rates, which has been documented by both increased carbon dioxide production, and by the penetration of toxins into tissues (Kodrík et al., 2015b). Thus, the intensity of the defence reaction is counter-productive and is subsequently fatal for insects.

There is an intensive effort to utilize insect neurohormones as biorational pesticides to reduce the number of chemical insecticides, to increase the efficacy of protection of plants and stored products, and to protect the environment (Borovsky and Nauen, 2007; Gäde and Goldsworthy, 2003; Verlinden et al., 2014). It seems that especially AKHs might be a good basis for further research (Kodrík et al., 2015b).

1.2.3. Adipokinetic hormones and their interactions with immune response

The involvement AKH in the insect immunity was firstly documented in *L. migratoria* (Goldsworthy et al. 2002a), the prophenoloxidase (ProPO) cascade in the hemolymph was activated after laminarin (an algal-derived immunogen fairly typical of the β-1,3-glucans present in fungal cell walls) injecting, and this activation was prolonged when Locmi-AKH-I was co-injected with the immunogen. Furthermore, the injection of bacterial lipopolysaccharide (LPS) from *Escherichia coli* did not enhance the phenoloxidase activity in the hemolymph but induced the formation of nodules. On the other hand, co-injection of Locmi-AKH-I and LPS increased the number of nodules being formed and activated also the ProPO cascade (Goldsworthy et al., 2003a, b). Additionally, co-injected AKH with conidia of *Metarhizium anisopliae* (by a topical application) or *Bacillus megaterium* (by injection) enhanced the mortality of locusts (Goldsworthy et al., 2005). Other studies detected changes in AKH receptor expression upon immune or heat challenges, suggest that the receptor does play a

role in physiological processes (Wang et al., 2012; Mayack et al 2015; Bordier et al., 2017).

Recently, it has been shown that AKH interacts also with a reaction induced by s venom from the parasitic wasp *Habrobracon hebetor* (Shaik et al., 2017). This venom elicits complete neuromuscular paralysis in insects (Beckage and Gelman, 2004; Sláma and Lukáš, 2011), and strongly suppresses cell and humoral immunity (Kryukova et al., 2007, 2011; Pennacchio et al., 2014). Both actions aim to immobilize the host ('a living can') and suppress its defense immune response, providing optimal conditions for the laid eggs and for the new generation of H. *hebetor*. Venom components interact with receptors on the presynaptic membranes of neuromuscular junctions, probably blocking glutaminergic transmission (Slavnova et al., 1987; Pennacchio and Strand, 2006). Studies on the cellular and humoral immune responses of insects parasitized by *H. hebetor* are scarce (Hartzer et al., 2005). Nevertheless, venom components might have a potential function in targeting the two major host defense effectors, i.e., phenoloxidase cascade, and coagulation (Beckage and Gelman, 2004; Andrew et al., 2006). Phenoloxidase activity was suppressed in wax moth *Galleria mellonella* larvae after venom application, but other components of the cell and humoral immunity were also affected (Kryukova et al., 2011, 2015).

1.3. Entomopathogenic nematodes

Entomopathogenic nematodes (EPNs) are obligate parasites completing their entire life cycle in insect hosts, which cause diseases and kill the insects (Shapiro et al., 2006). They are mass- produced and marketed worldwide as biocontrol agents for the control of a wide range of insect species of agricultural or medical importance (Ehlers, 2011).

The main advantages and disadvantages of EPNs for control of insect pests are (Shapiro-Ilan et al., 2012):

Advantages	Disadvantages
- Broad pest insect host range - Rapid speed of kill - Actively seek or ambush host - Mass in vivo and in vitro production capability - Possible to use conventional application equipment - Safety for all vertebrates, most non-target invertebrates, and the food supply - Little or no registration required	- Cost of production - Limited shelf-life and refrigerated storage required - Environmental limitations: requirements for adequate moisture (to enable survival and infectivity) and temperatures (above or below that required for optimal infectivity), sensitivity to UV radiation, lethal effect of several pesticides (nematicides, fumigants and others), lethal or restrictive soil chemistries (high salinity, high or low pH, etc.).

Like many other nematode parasites, EPNs live in symbiotic relationship with bacteria that help them to kill host insects and bio-convert its tissues to produce nutrients for emerging generations of EPN. In particular, the bacteria are found in

the gut of the infective juvenile (IJ) worms that are able to attack and invade insects (Ciche and Ensign, 2003). EPNs have been described from 23 nematode families (Koppenhofer, 2007), those belonging to the family's Steinemernatidae and Heterorhabditidae have received the most attention because they possess many of the attributes of effective biological control agents (Kaya and Gaugler, 1993; Grewal et al., 2005; Koppenhofer, 2007). These two nematode families carry symbiotic *Xenorhabdus* and *Photorhabdus* bacteria, respectively, which are also pathogens of insects. The mutualistic relationship between nematodes and their bacteria is not obligated, as nematodes can kill the host in the absence of their mutualistic bacteria (Herbert and Goodrich-Blair, 2007; Waterfield et al., 2009).

The general characteristics of the life cycles of these nematodes are relatively similar. EPNs complete most of their life cycle within the insect body with an exception of IJs, the only free-living stage found in soil. The IJs are able to survive in soil and are required for successful infection of insect hosts (Goodrich-Blair, 2007; Waterfield et al. 2009). The infection starts with a search for the hosts, followed by the attachment of the nematodes to the cuticle and penetration through natural openings (mouth, anus, spiracles, and tracheae). Then both the nematode and bacteria must overcome the host's natural barriers – including the peritrophic membrane, intestinal wall and adjacent tissues and invade the insect hemocoel to become established in the hemolymph.

Inside the host, the nematodes finish development into adult hermaphrodite worms and start to lay eggs, which hatch and develop through four larval stages into the next generation of adult nematodes. The cycle continues, reproducing two to three generations before forming the IJ stage when the food is depleted within the insect cadaver. After that IJ nematodes emerge from the insect carcass into the soil to seek new suitable hosts (Silva et al., 2002; Waterfield et al., 2009). Within the life cycle, the nematodes release their symbiotic bacteria which rapidly multiply and kill the host within 2–3 days (Krecek and Waller, 2006) by secretion of several toxins and virulence factors that cause septicemia in the midgut and fat body tissues.

Under optimal laboratory conditions, approximately 100 000 IJ nematodes can be produced from a single IJ infecting an individual insect. Owing to their complex life cycles and close association with the virulent bacterial pathogens *Photorhabdus* and *Xenorhabdus*, the *Heterorhabditis* and *Steinernema* nematodes are used in basic research providing a fascinating model for the study of insect immunity and disease progression, bacterial pathogenicity/symbiosis and nematode parasitism (Eleftherianos et al., 2010).

Insect hosts defend themselves against the invasion by EPNs with highly potent cellular immune (include phagocytosis and encapsulation) and humoral immune (include inducible anti-microbial peptides, cell adhesion molecules, lysozymes, lectins and the ProPO system) responses to eliminate or reduce an infection (Castillo et al., 2011). These responses locally, systemically and together contribute to protection against attacks by foreign microorganisms (Uvell et al., 2007).

EPNs and their symbionts employ various strategies to actively destroy or manipulate immune insect defense mechanisms (Gotz et al., 1981). The whole complex produces virulence factors which are mostly proteases (Li et al., 2007), phenoloxidase inhibitors and toxins that interfere for example with phagocytosis (Sicard et al., 2008; Hao et al., 2008). The secreted proteases facilitate EPN penetration into the host hemocoel and counteract the insect defense system (Kaya and Gaugler, 1993). Additionally, EPNs can escape insect defenses due to surface proteins and lipids (Brivio et al., 2004), which are not recognized by insect defense cells thanks to secreted chymotrypsin proteases. Those proteases are also able to destroy antibacterial peptides, expressed by insects in response to parasitism (Gotz et al., 1981). Another strategy by EPN symbiotic bacteria has been found to evade the cellular immune response by killing hemocytes and by employing mechanisms that suppress key cellular responses in insects (Eleftherianos et al. 2010).

Immune evasion strategies of parasites can be divided into three main groups:

A. Anatomical seclusion: involves the migration of the parasites to host tissues where they can avoid immune reactions, such as encapsulation by hemocytes.
B. Camouflage strategy: involves the production of mimicking molecules or the sequestration of immune factors from the host hemolymph. These molecules are deposited onto the body surface of the parasite and help to avoid recognition and induction of host immune responses.
C. Interference strategy: involves the suppression, disruption or modulation of host immune defenses (humoral or cellular) by the parasite.

1.4. Immune response in insects

In general, animal immunity can be divided into innate and adaptive. The innate immunity is characterized as a general or nonspecific immune response which is conserved across all organisms, including insects and responds similarly against a repeated challenge (Medzhitov and Janeway, 2000). The innate immunity is the first line defense that can act fast and often effectively to control and eliminate pathogens such as bacteria, viruses, fungi, protozoans and parasites. The adaptive immunity is characterized by two key traits. First, it can remember the pathogen it has received before, thus, the defense response can be quicker during a subsequent infection. Second, it is able to mount a stronger defense, which is highly specific and contains mechanisms targeting to a particular microbe during a new encounter, suggesting that the defense is specific to that elicitor (Litman et al., 2010). Insects lack an adaptive immune system and rely only on innate immunity. The innate immune response in insects is more complex and involves mechanisms such as physical barriers, cellular responses, and humoral responses (Müller et al., 2008).

Physical barriers that prevent the entry of pathogens include integument, peritrophic membrane and trachea. Integument or exoskeleton, the external surface of an insect, is a chitinous, hydrophobic material, ectodermal in origin, formed by a single layer of cells covered by a multilayered cuticle (Ashida and Brey, 1995). Integument represents a significant barrier to insect pathogens.

The peritrophic membrane layer is permeable to nutrients and enzymes, made of chitin and glycoprotein that lines the insect midgut. It affords physical protection to the delicate midgut epithelium against abrasive food particles and digestive pathogens (Richards and Richards, 1977; Peters, 1992).

The spiracles and the whole tracheal system are one of the most important potential sites of the pathogen's entry into an insect host (Doucet et al., 1998). This route is commonly used by nematodes and bacterial and fungal spores. Despite the potential ease of entry via this route, there is very little information about how insects avoid infection through it. However, there are studies showing that the epithelium associated with the trachea is immunologically active (Tzou et al., 2000) and that there are intimate spatial relationships between hemocytes and trachea (Wigglesworth, 1965) suggestive of a defensive role.

Humoral and cellular immune responses in insects are induced after microorganisms entering these barriers to their hemocoel; the fat body and hemocytes are mostly responsible for the response initiation (Medzhitov and Janeway, 1997). Upon infection of the hemocoel, cellular immune responses are involved almost immediately by nodulation, encapsulation, and phagocytosis; external wounding is usually followed by clot formation (Sideri et al., 2007). On the other hand, humoral responses are much slower; it takes usually hours than this system is activated. Humoral responses include production of antimicrobial peptides (Hoffmann, 2003), which are synthesized predominantly in fat body and released into hemolymph (Shia et al., 2009), activation of ProPO that regulate coagulation and melanization of hemolymph (Nappi and Vass, 2001), and production of reactive oxygen and nitrogen species (Mavrouli et al., 2005).

1.4.1. Insect cellular immune response

Cellular defense responses in insects are well known for about 100 years. The primary immune cells in insects are the hemocytes that are traditionally identified by microscopic observations using morphological, histochemical and functional characteristics. The most common types of circulating hemocytes reported in the literature are prohemocytes, granular cells (granulocytes) that drive phagocytosis, plasmatocytes that are involved in capsule formation, spherule cells (spherulocytes) whose immune function remains unclear, and oenocytoids that produce enzymes involved in the melanization cascade such as phenoloxidase (Lavine and Strand 2002; Meister and Lagueux, 2003; Lamprou et al., 2007; Strand, 2008). These hemocyte types are not present in all insect species.

The primary defense responses of hemocytes to pathogens are phagocytosis, nodulation and encapsulation:

1.4.1.1. Phagocytosis

Phagocytosis is a complex process referring to the engulfment of microbes by an individual cell. It requires expression of large numbers of genes, and it is probably the oldest defense mechanism for rapid removal of dead cells during infections, but also during metamorphosis or embryogenesis. Interestingly, in the mosquito *A. aegypti* and tobacco hornworm *Manduca sexta*, hundreds of bacteria can be phagocytosed by hemocytes at any given time (Dean et al., 2004; Hillyer

et al., 2005). Phagocytosis involves actin filaments and can be blocked by cytochalasin B (Scapagliati and Mazzini, 1994; Wago, 1991). Hemocyte phagocytosis rate can be increased by factors of microbial origin such as glucans and may also be stimulated by the ProPO-activating cascade (Johansson and, Söderhäll, 1992). Hemocytes phagocyte both biotic and abiotic targets like bacteria, yeast, apoptotic bodies and synthetic beads or particles of India ink (Yokoo et al., 1995; Hernandez et al., 1999; de Silva et al., 2000b).

The hemocytes that phagocyte pathogens are reported to be phagocytic varies among insect taxa, in Lepidoptera, Hemiptera and mosquitoes are the granulocytes, while in the fruit fly *Drosophila* the plasmatocytes are the main phagocytic hemocyte (Hillyer et al., 2003a, b; Hillyer and Strand, 2014; Honti et al., 2014; Laughton et al., 2011; Strand, 2008). As a percentage of the overall hemocyte population, the majority of hemocytes are phagocytic.

Digestion of microbes by phagocytic cells is an extremely complex, diverse process, and it seems that several distinct molecular mechanisms regulate it.

1.4.1.2. Nodulation

Nodules are multicellular hemocytic aggregates that surround a large group of bacteria. The whole mechanism is activated by hemocytes when the initial phagocytic immune response is not sufficient. The nodulation process is completed by covering with layers of flattened hemocytes to isolate bacteria from the hemolymph; this is usually followed by melanization (Ratcliffe and Gagen, 1977; Satyavathi et al., 2014). In many insect species, this immune process is not completely characterized, but certain molecules such as eicosanoids, ProPO or dihydroxyphenyl alanine (DOPA) decarboxylase also participates (Sideri et al., 2007).

1.4.1.3. Encapsulation

Insects also express an additional form of cellular immunity to invasion by pathogens larger than bacterial cells, such as eggs or larvae of parasitic wasps (parasitoids), protozoa, and nematodes, by surrounding them within multiple hemocytes (Nappi et al., 1995). The hemocytes usually involved in encapsulation are granulocytes and plasmatocytes in Lepidoptera, but in *Drosophila*, the cells involved are plasmatocytes and lamellocytes (Russo et al., 1996). Hemocytes are surrounding to their target and forming a multiple layer capsule around the invader, which is usually followed by melanization (Nappi and Ottaviani, 2000). Inside the capsule, the encapsulated organism almost always dies by the local production of several factors including asphyxiation, reactive cytotoxic products and antibacterial peptides (Carton et al., 2009).

1.4.2. Insect humoral immune responses

There is considerable overlap between humoral and cellular defenses in processes like the recognition of invading pathogens such as bacteria, fungi and viruses. Insect humoral immunity involves processes like activation of enzymatic cascades that regulate coagulation and melanization of hemolymph, production of reactive oxygen and nitrogen species (Gilespie et al., 1997; Bogdan et al., 2000;

Nappi and Vass, 2001; Hoffmann, 2003; Mavrouli et al., 2005), and the secretion of antimicrobial peptides (Hoffmann, 2003)). Due to the complexity of the insect humoral immunity just the antimicrobial peptides (AMPs) will be referred in detail.

1.4.2.1. Antimicrobial peptides

Production of AMPs is highly inducible: their rate of synthesis is multiplied upon a microbial infection, and their level in hemolymph raises sharply, while in uninfected animals the AMP level is practically undetectable (Zasloff, 2002; Bulet et al., 2004). AMPs are mainly synthesized and expressed by the fat body, and to a lesser extent by the hemocytes, integument, gut, salivary glands and reproductive structures (Marmaras and Lampropoulou, 2009; Jiang et al., 2010). In insect species, over 150 AMPs have been found, isolated and identified (Yi et al., 2014). The first antimicrobial protein that has been characterized was the lysozyme in *G. mellonella*. Most AMPs are small, cationic proteins able to degrade bacterial cell wall peptidoglycans of gram-positive bacteria. They also show some activity against gram-negative bacteria and against some fungi leading to disruption and cell death (Chapelle et al., 2009; Wang et al., 2009).

Insect AMPs can be classified based on their structure or function. They are divided to three major structural classes a) linear α-helical peptides without cysteine residues (e.g. cecropin and moricin), b) peptides with a β-sheet globular structure stabilized by intramolecular disulfide bridges (e.g. insect defensin and drosomycin), and c) peptides that contain unusually high numbers of specific amino acid residues, such as proline (e.g. apidaecin, drosocin, and lebocin) or glycine (e.g. attacin and gloverin) (Bulet et al., 2004; Bulet and Stocklin, 2005; Wiesner and Vilcinskas 2010). The functional classification of insect AMPs tends to be based on the target pathogen range rather than any specific mechanism of action. Some AMPs have a broad range, whereas others show varying degrees of specificity towards gram-positive or gram-negative bacteria, fungi, parasites and even viruses (Pretzel et al., 2013). Even AMPs from the same class, but different insect species may have activity against different microorganisms depending on its binding ability to the microorganism (Yi et al., 2014).

Recently, huge progress has been made in the study of the natural immunity of insects by using the fruit fly as a model organism. Together seven antibacterial peptides have been identified here: cecropin, attacin, defensin, drosocin, diptericin, metchnikowin and also drosomycin (Lemaitre and Hoffmann, 2007). Toll and immune deficiency signaling pathways which regulate *Drosophila* AMP genes have been well studied.

1.4.2.2. Role of adenosine in defense reactions

Adenosine plays an important role in physiological regulation both inside and outside the cell of vertebrates and invertebrates. It is a purine nucleoside, derived primarily from the dephosphorylation of adenine nucleotides (Fig. 1), a paracrine and systemic signalling molecule of metabolic imbalance.

Fig. 1. Structure of adenosine

Adenosine accumulates extracellularly in response to cell damage, hypoxia, parasitization or metabolic stress (Csóka and Haskó, 2011), by dephosphorylation of ATP, ADP and AMP. The reaction is catalyzed by ecto-nucleoside triphosphate diphosphorylases and by ecto-5′-nucleotidase. The adenosine concentration is controlled by its conversion into inosine (Yegutkin, 2008). Intracellular adenosine is exported from cells via equilibrative nucleoside transporters through facilitated diffusion that efficiently balances between the intra- and extracellular levels of adenosine (Fig. 2).

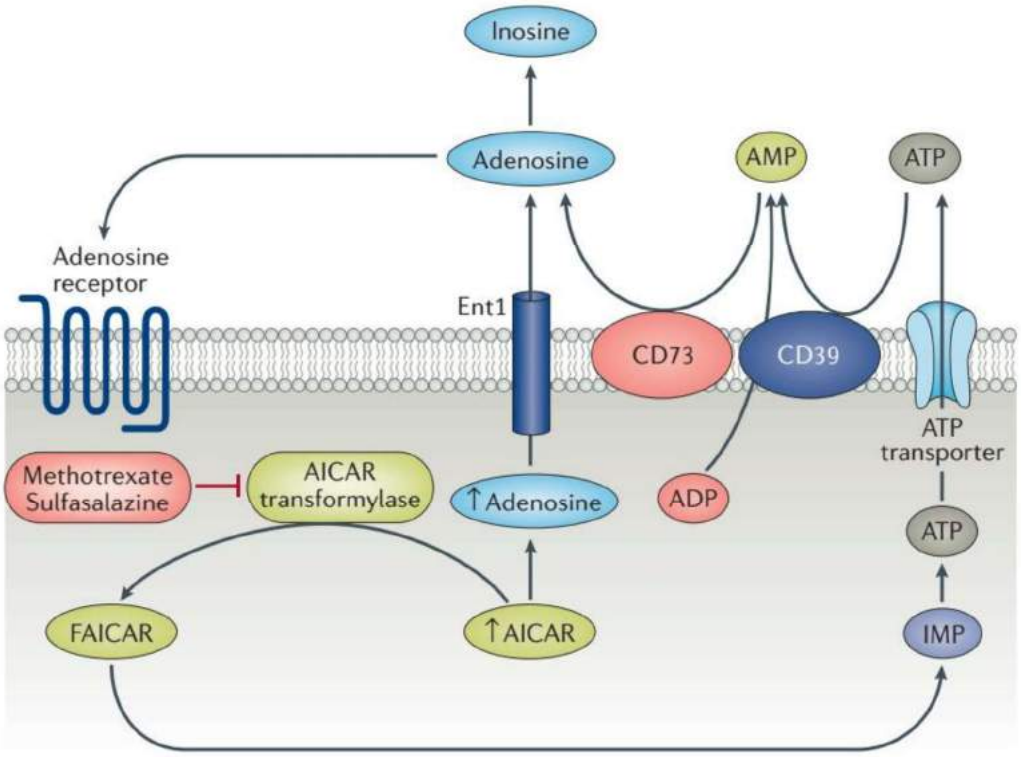


Fig. 2 Cellular adenosine production in response to hypoxia and medications. Adenosine is produced extracellularly from the hydrolysis of adenine nucleotides (ATP, ADP and AMP) by the ectoenzymes nucleoside triphosphate phosphohydrolase (NTPP or CD39) and ecto-5′nucleotidase (CD73), whereas adenosine levels in the extracellular space are reduced by cellular uptake via the extracellular nucleoside transporter (Ent1) and breakdown extracellularly to inosine by adenosine deaminase. Both methotrexate and sulfasalazine diminish the activity of 5-aminoimidazole-4 carboxamide ribonucleotide (AICAR) transformylase leading to accumulation of AICAR and reduction of its metabolite formyl AICAR (FAICAR). Intracellular accumulation of AICAR leads to increased ATP release into the extracellular space. Inosine monophosphate (IMP) is the final product of de novo purine biosynthesis and can be converted further to adenine and guanine nucleotides. Ultimately, much of the IMP is converted to ATP, which can be released into the extracellular space as well (Cronstein and Sitkovsky, 2017).

According to its mode of action, adenosine is also considered to be a stress 'hormone' comparable with typical hormones (Haskó, 2002). Adenosine is one of the intercellular signals maintain homeostasis in tissues and organs, it is involved in regulating energy metabolism, many physiological and pathological processes, antioxidation, immune response, adipose tissue formation, and neural functions (Jacobson and Gao, 2009). Adenosine regulates cellular functions by binding to its receptors (AdoR) which belong to the G protein-coupled receptor (GPCR) family (Fredholm et al., 2000; Csóka and Haskó, 2011). In *D. melanogaster* a single AdoR has been identified; the receptor belongs to the stimulatory subclass

of G proteins and its activation regulates intracellular cAMP (Dolezelová et al., 2007; Kucerová et al., 2012).

Moreover, during immune responses, extracellular adenosine released from immune cells mediates a systemic metabolic switch leading to change the growth of wing disc and energy storage, thus leaving a huge amount of energy is available to use by the immune cells (Bajgar et al., 2015).

Thus, it is evident there are significant similarities between adenosine and AKH. These similarities involve regulatory functions on physiological and biochemical levels such as energy metabolism (mobilization of nutrients) but include also neural functions, participation in immune response, and control of physiology of adipose tissue and anti-oxidation stress reactions (Jacobson and Gao, 2009; Zemanová et al., 2016). In addition, on a cellular level, both adenosine and AKH signaling pathways overlap in employing identical pathways. For example, GPCRs mediate both pathways through modulating cAMP production (Park et al., 2002; Jacobson and Gao, 2009).

1.5. Vitellogenin

Vitellogenins (Vgs), are glycolipophosphoproteins, defined and well-characterized as the predominant precursors of yolk proteins in insects and many other oviparous animals. In most insects, Vg is produced extra-ovarially in the fat body. From the fat body, they are transported via hemolymph, and then taken up by the growing oocytes through receptor-mediated endocytosis against the concentration gradient (Chapman, 1998). In ovaries, the Vg are transformed to vitelline, the main nutritional reserve for the developing embryo (Chapman, 1998). Several studies revealed that Vg occurs also in males: the list of such insect species includes also *P. apterus* (Němec et al., 1993).

Most insect species synthesize only one or two types of Vg molecules that comprise of several polypeptides with the total molecular weight ranging from 150 to 650 kDa. Vg synthesis is hormonally controlled at the transcriptional level. The main stimulatory role is played by juvenile hormones, but in some species, ecdysteroids are involved as well (Fig. 3). On the other hand, AKH inhibits synthesis of the protein part of the Vg molecule directly in the fat body (Carlisle and Loughton, 1986). In addition, change in Vg synthesis is also controlled by the nutrient levels and mating status (Chapman, 1998).

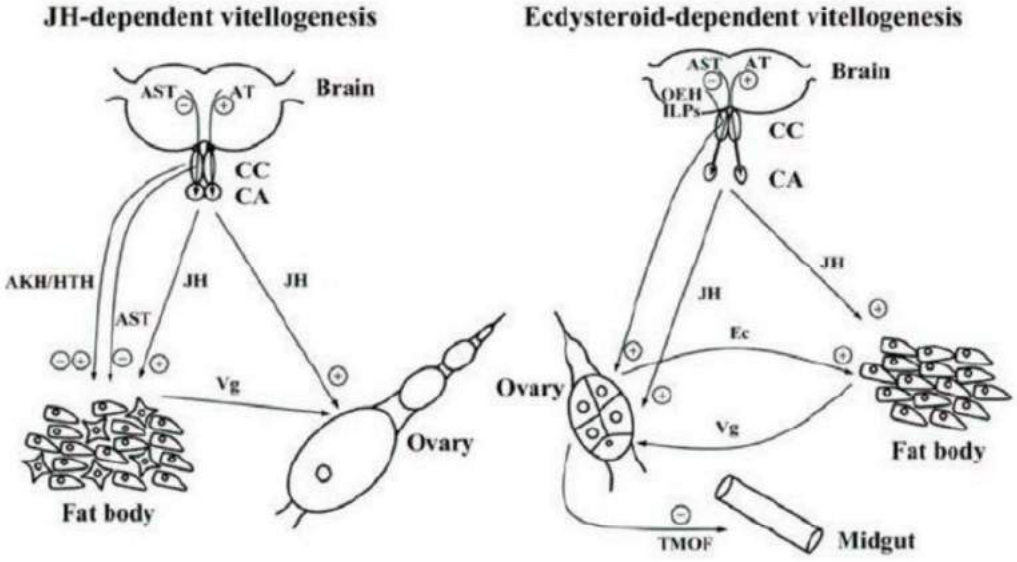


Fig. 3. Hormones influencing vitellogenesis in JH- and ecdysteroid dependent insects. AKH: adipokinetic hormone; AST: allatostatin; AT: allatotropin; CA: corpora allata; CC: corpora cardiaca; Ec: ecdysteroids; HTH: hypertrehalosemic hormone; ILPs: insulin-like peptides; JH: juvenile hormone; OEH: ovarian ecdysteroidogenic hormone; TMOF: tripsin-modulating oostatic factor; Vg: vitellogenin. Signs + and - indicate influence on vitellogenesis (Belles and Maestro, 2005).

It is well documented that Vg have also other functions inside the insect body such as control of caste differentiation process in social insects (Salmela and Sundstrom, 2017), wound healing (Park et al., 2018), protection against OS (Havukainen et al., 2013; Park et al., 2018), role in immunity (Singh et al., 2013; Salmela et al., 2015), and life span regulation (Havukainen et al., 2013; Salmela and Sundstrom, 2017). Several studies revealed that Vg molecules 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). In addition, the infection by the spores of the microsporidium *Nosema ceranae* significantly up-regulated Vg expression in honey bee workers (BenVau and Nieh, 2017; Sinpoo et al., 2018). Thus, Vgs seems to be important players in insect immune response similarly as e.g. antimicrobial peptides.

2. References

Ahmad S. (1995) Oxidative stress from environmental pollutants. Arch. *Insect Biochem. Physiol.* 29:135–157.

Ahmad S., Beilstein M., A., Pardini R., S. (1989) Glutathione peroxidase activity in insects: A reassessment. *Arch. Insect Biochem. Physiol.* 12: 31-49.

Alves-Bezerra M., De Paula I.F., Medina J.M., Silva-Oliveira G., Medeiros J.S., et al. (2015) Adipokinetic hormone receptor gene identification and its role in triacylglycerol metabolism in the blood-sucking insect *Rhodnius prolixus*. *Insect Biochem. Mol.* 69: 51-60.

Andrew N., Basio M., Kim Y. (2006) *Additive effect of teratocyte and calyx fluid from Cotesia plutellae* on immunosuppression of *Plutella xylostella*. *Physiol. Entomol.* 31: 341–347.

Ashida M., Brey P.T. (1995) Role of the integument in insect defense: prophenol oxidase cascade in the cuticular matrix. *Proc. Natl. Acad. Sci. USA.* 92: 10698–10702.

Bajgar A., Kucerová K., Jonátová L., Tomcala A., Schneedorferová I., Okrouhlík J.,
Dolezal T. (2015) Extracellular adenosine mediates a systemic metabolic switch
during immune response. *PLoS Biol.* 13: e1002135.

Beckage N.E., Gelman D.B. (2004) Wasp parasitoid disruption of host development: implications for new biologically based strategies for insect control. *Annu. Rev. Entomol.* 49: 299–330.

Bednářová A., Kodrík D., Krishnan N. (2013a) Unique roles of glucagon and glucagon-like peptides: Parallels in understanding the functions of adipokinetic hormones in stress responses in insects. *Comp. Biochem. Physiol. A Mol. Integr. Physiol.* 164: 91–100.

Bednářová A., Kodrík D., Rakshit K., Krishnan N. (2015) Knockdown of adipokinetic hormone synthesis increases susceptibility to oxidative stress in *Drosophila* – a role for dFoxO? *Comp. Biochem. Physiol. C* 171: 8-14.

Bednářová A., Krishnan N., Cheng I.C., Večeřa J., Lee H.J., Kodrík D. (2013b) Adipokinetic hormone counteracts oxidative stress elicited in insects by hydrogen peroxide: in vivo and in vitro study. *Physiol. Entomol.* 38: 54–62.

Belles X., Maestro J.L. (2005) Endocrine peptides and insect reproduction. *Invertebr. Reprod. Dev.* 47: 23-37.

Belmont M., Cazzamali G., Williamson M., Hauser F., Grimmelikhuijzen C.J. (2006) Identification of four evolutionarily related G protein-coupled

receptors from the malaria mosquito *Anopheles gambiae*. *Biochem Biophys Res. Commun*. 344(1): 160-5.

BenVau L.R., Nieh J.C. (2017) Larval honey bees infected with *Nosema ceranae* have increased vitellogenin titers as young adults. *Sci. Rep*. 7: article number 14144.

Bi J.L., Felton G.W. (1995) Foliar oxidative stress and insect herbivory: Primary compounds, secondary metabolites, and reactive oxygen species as components of induced resistance. *J. Chem. Ecol*. 21: 1511–1530.

Bil M., Broeckx V., Landuyt B., Huybrechts R. (2014) Differential peptidomics highlights adipokinetic hormone as key player in regulating digestion in anautogenous flesh fly, Sarcophaga crassipalpis. *Gen. Comp. Endocrinol*. 208: 49-56.

Bil M., Timmermans I., Verlinden H., Huybrechts R. (2016) Characterization of the adipokinetic hormone receptor of the anautogenous flesh fly, *Sarcophaga crassipalpis*. *J. Insect Physiol*. 89: 52-9.

Bodláková K., Beňová M., Kodrík D. (2018) The effect of adipokinetic hormones on the activity of digestive enzymes. *Physiol. Entomol*. 43: 140-148.

Bodláková K., Jedlička P., Kodrík D. (2017) Adipokinetic hormones control amylase activity in the cockroach (*Periplaneta americana*) gut. *Insect Sci*. 24: 259-269.

Bordier C., Suchail S., Pioz M., Devaud J.M., Collet C., Charreton M., Le Conte Y., Alaux C. (2017) Stress response in honeybees is associated with changes in task-related physiology and energetic metabolism. *J. Insect Physiol*. 98: 47-54.

Borovsky D., Nauen R. (2007) Biological and biochemical effects of organo-synthetic analogues of Trypsin Modulating Oostatic Factor (TMOF) on *Aedes aegypti*, *Heliothis virescens* and *Plutella xylostella*. *Pestic*. 3-4: 17-26.

Bray M.M., Shafi S., Wheeler C.H., Goldsworthy G.J. (1993) Quantification by radioimmunoassay of the adipokinetic hormoneI in neural tissues in the head of *Locusta migratoria*. *Comp. Biochem. Physiol*. 106A: 257 – 262.

Brivio M.F., Mastore M., Moro M. (2004) The role of *Steinernema feltiae* body surface lipids in host-parasite immunological interactions. *Mol. Biochem. Parasitol*. 135: 111–121.

Bulet P., Stocklin R. (2005) Insect antimicrobial peptides: structures, properties and gene regulation. *Protein Pept. Lett*. 12: 3–11.

Bulet P., Stocklin R., Menin L. (2004) Anti-microbial peptides: from invertebrates to vertebrates. *Immunol. Rev*. 198: 169-184.

Burlakova L.E., Padilla D.K., Karatayev A.Y., Hollas D.N., Cartwright L.D., Nichol K.D. (2010) Differences in population dynamics and potential

impacts of a freshwater invader driven by temporal habitat stability. *Biol Invasions. Vol.* 12. 4: p927.

Caers J., Janssen T., Van Rompay L., Broeckx V., Van Den Abbeele J., Gäde G., Schoofs L., Beets I. (2016) Characterization and pharmacological analysis of two adipokinetic hormone receptor variants of the tsetse fly, *Glossina morsitans morsitans*. *Insect Biochem. Mol. Biol.* 70: 73-84.

Carlisle J., Loughton B.G. (1979) Adipokinetic hormone inhibits protein synthesis in locusta. *Nature.* 282, 420-421.

Carton Y., Frey F., Nappi A.J. (2009) Parasite induced changes in nitric oxide levels in Drosophila paramelanica. *J. Parasitol.* 95: 1134-1141.

Castillo J.C., Reynolds S.E., Eleftherianos I. (2011) Insect immune responses to nematode parasites. *Trends Parasitol.* 27: 537-47.

Chapelle M., Girard P.A., Cousserans F., Volkoff N.A., Duvic B. (2009) Lysozymes and lysozymelike proteins from the fall armyworm, *Spodoptera frugiperda*. *Mol. Immunol.* 47: 261-269.

Chapman R.F. (1998) The Insects, Structure and Function. *Cambridge, UK: Cambridge University Press.*

Ciche T.A., Ensign J.C. (2003) For the insect pathogen *Photorhabdus luminescens*, which end of a nematode is out? *Appl. Environ. Microbiol.* 69: 1890-7.

Clottens F., Gade G., Huybrechts R., de Loof A. (1989) Immunohistochemical localisation of the hypertrehalosaemic hormone II (Cam-HrTH-II) and related peptides in the nervous system of *Carausius morosus* and *Sarcophaga bullata. Cell Tissue Res.* 258: 631-636.

Cronstein B.N., Sitkovsky M. (2017) Adenosine and adenosine receptors in the pathogenesis and treatment of rheumatic diseases. *Nat Rev Rheumatol.* 13: 41–51.

Csóka B., Haskó G. (2011) Adenosine, inflammation pathways and therapeutic challenges. *Joint Bone Spine.* 78: 4–6.

de Doucet M.M.A., Miranda M.B. Bertolott, M.A. (1998) Infectivity of entomogenous nematodes (*Steinernematidae and Heterorhabditidae*) to *Pediculus*
humanus capitis De Geer (Anoplura: Pediculidae). *Fund. Appl. Nematol.* 21: 13–16.

de Silva J.B., de Albuquerque C.M.R., de Araujo E.C., Peixoto C.A., Hurd H.H. (2000b) Immune defense mechanisms of *Culex quinquefasciatus* (Diptera: Culicidae) against *Candida albicans* infection. *J. Invert. Pathol.* 76: 257-262.

Dean P., Potter U., Richards E.H., Edwards J.P., Charnley A.K., Reynolds S.E. (2004) Hyper phagocytic hemocytes in *Manduca sexta*. *J. Insect Physiol*. 50: 1027-1036.

Dolezelova E., Nothacker H.P., Civelli O., Bryant P.J., Zurovec M. (2007) A *Dr osophila* adenosine receptor activates cAMP and calcium signaling. *Insect Biochem. Mol. Biol.* 37: 318–329.

Ehlers R.U. (2001) Mass production of entomopathogenic nematodes for plant protection. *Appl. Microbiol. Biotechnol*. 56: 523-633.

Eleftherianos I., ffrench-Constant R.H., Clarke D.J., Dowling A.J., Reynolds S.E. (2010) Dissecting the immune response to the entomopathogen Photorhabdus. *Trends Microbiol.* 18: 552–560.

Farr S.B., Kogoma T. (1991) Oxidative stress responses in *Escherichia coli* and *Salmonella typhimurium*. *Microbiol. Mol. Biol. Rev.* 55: 561–585.

Felton G.W., Summers C.B. (1995) Antioxidant systems in insects. *Arch. Insect Biochem. Physiol.* 29: 187–197.

Fredholm B. B., et al. (2000) Structure and function of adenosine receptors and their genes. *NaunynSchmiedeberg's Arch. Pharmacol*. 362: 364-374.

Gade G. (2009) Peptides of the adipokinetic hormone/red pigment-concentrating hormone family: A new take on biodiversity. *Ann. NY. Acad. Sci.* 1163: 125-136.

Gade G., Goldsworthy G.J. (2003) Insect peptide hormones: a selective review of their physiology and potential application for pest control. *Pest Manag. Sci.* 59: 1063–1075.

Gade G., Hoffmann K.H., Spring J.H. (1997) Hormonal regulation in insects: facts, gaps, and future directions. *Physiol. Rev.* 77: 963–1032.

Gilespie J.P., Kanost M.R., Trenczek T. (1997) Biological mediators of insect immunity. *Annu. Rev. Entomol*. 42: 611-643.

Goldsworthy G.J., Chandrakant S., Opoku-Ware K. (2003a) Adipokinetic hormone enhances nodule formation and phenoloxidase activation in adult locusts injected with bacterial lipopolysaccharide. *J. Insect Physiol*. 49(8): 795-803.

Goldsworthy G.J., Opoku-Ware K., Mullen L.M. (2002) Adipokinetic hormone enhances laminarin and bacterial lipopoly saccharide-induced activation of the prophenoloxidase cascade in the African migratory locust, *Locusta migratoria*. *J. Insect Physiol*. 48: 601-608.

Goldsworthy G.J., Opoku-Ware K., Mullen L.M. (2005) Adipokinetic hormone and the immune responses of locusts to infection. *Annu.* N. Y. *Acad. Sci.* 1040: 106-113.

Goodrich-Blair H. (2007) they've got a ticket to ride: *Xenorhabdus nematophila-Steinernema carpocapsae* symbiosis. *Curr. Opin. Microbiol.* 10: 225–230.

Gotz P., Boman A., Boman H.G. (1981) Interaction between insect immunity and an insect- pathogenic nematode with symbiotic bacteria. *Proc. R. Soc. Lond.* 212: 333–350.

Grewal P.S., Ehlers R.U., Shapiro-Ilan D.I. (2005) Nematodes as biological control agents. *CABI Publishing, Wallingford, UK*

Hagedorn H.H., Maddison D.R., Tu Z. (1998) The evolution of vitellogenins, cyclorrhaphan yolk proteins and related molecules. *Advances in Insect Physiology*. 27: 335-384.

Hansen K.K., Hauser F., Cazzamali G., Williamson M., Grimmelikhuijzen C.J. (2006) Cloning and characterization of the adipokinetic hormone receptor from the cockroach *Periplaneta americana*. *Biochem Biophys Res Commun*. 343(2): 638-43.

Hansen K.K., Stafflinger E., Schneider M., Hauser F., Cazzamali G., Williamson M., Kollmann M., Schachtner J., Grimmelikhuijzen C.J. (2010) Discovery of a novel insect neuropeptide signalling system closely related to the insect adipokinetic hormone and corazonin hormonal systems. *J Biol Chem*. 285(14): 10736-47.

Hao Y.J., Montiel R., Nascimento G., Toubarro D., Simoes N. (2008) Identification, characterization of functional candidate genes for host-parasite interactions in entomopathogenetic nematode *Steinernema carpocapsae* by suppressive subtractive hybridization. *Parasitol Res*. 103: 671–683.

Hartzer K.L., Zhu K.Y., Baker J.E. (2005) Phenoloxidase in larvae of Plodia interpunctella (Lepidoptera: Pyralidae): Molecular cloning of the proenzyme cDNA and enzyme activity in larvae paralyzed and parasitized by Habrobracon hebetor (Hymenoptera: Braconidae). Arch. *Insect Biochem. Physiol*. 59: 67-79.

Haskó G. (2002) Adenosine: a potential mediator of immunosuppression in multiple
organ failure. *Curr. Opin. Pharmacol*. 2: 440–444.

Hauser F., Cazzamali G., Williamson M., Blenau W., Grimmelikhuijzen C.J. (2006) A review of neurohormone GPCRs present in the fruitfly *Drosophila melanogaster* and the honey bee *Apis mellifera*. *Prog Neurobiol*. 80(1):1-19.

Havukainen H., Münch D., Baumann A., Zhong S., Halskau Ø., Krogsgaard M., Amdam G.V. (2013) Vitellogenin recognizes cell damage through membrane binding and shields living cells from reactive oxygen species. *J. Biol. Chem*. 288: 28369-28381.

Herbert E.E., Goodrich-Blair H. (2007) Friend and foe: the two faces of *Xenorhabdus nematophila*. *Nat. Rev. Microbiol.* 5: 634–646.

Hernandez S., Lanz H., Rodriguez M.H., Torres J.A., Martinez P.A., Tsutsumi V. (1999.) Morphological and cytochemical characterization of female *Anopheles albimanus* (Diptera: Culicidae) hemocytes. *J. Med. Entomol.* 36: 426–434.

Hightower L.E. (1991) Heat shock, stress proteins, chaperones, and proteotoxicity. *Cell.* 66: 191-197.

Hillyer J.F., Christensen B.M. (2005) Mosquito phenoloxidase and defensin colocalize in melanization innate immune responses. *J Histochem Cytochem.* 53: 689-698.

Hillyer J.F., Schmidt S.L., Christensen B.M. (2003a) Hemocyte-mediated phagocytosis and melanization in the mosquito *Armigeres subalbatus* following immune challenge by bacteria. *Cell Tissue Res.* 313: 117–127.

Hillyer J.F., Schmidt S.L., Christensen B.M. (2003b) Rapid phagocytosis and melanization of bacteria and Plasmodium sporozoites by hemocytes of the mosquito *Aedes aegypti*. *J. Parasitol.* 89: 62-69.

Hillyer J.F., Strand M.R. (2014) Mosquito hemocyte-mediated immune responses. *Curr. Opin. Insect. Sci.* 3: 14-21.

Hoffmann JA. (2003) The immune response of *Drosophila*. *Nature*. 426: 33-38.

Honti V., Csordas G., Kurucz E., Markus R., Ando I. (2014) The cell-mediated immunity of *Drosophila melanogaster*: hemocyte lineages, immune compartments, microanatomy and regulation. *Dev. Comp. Immunol.* 42: 47–56.

Huang H., He X., Deng X., Li G., Ying G., Sun Y., Shi L., Benovic J.L., Zhou N. (2010). Bombyx adipokinetic hormone receptor activates extracellular signal-regulated kinase 1 and 2 via G protein-dependent PKA and PKC but β-arrestin-independent pathways. *Biochemistry*. 49(51):10862-72.

Huang J.H., Belles X., Lee H.J. (2012) Functional characterization of hypertrehalosemic hormone receptor in relation to hemolymph trehalose and to oxidative stress in the cockroach *Blattella germanica*. *Front. Endocrinol.* 2: 114 (1-11).

Ibrahim E., Hejníková M., Shaik H.A., Doležel D., Kodrík D. (2017) Adipokinetic hormone activities in insect body infected by entomopathogenic nematode. *J. Insect*
Physiol. 98: 347–355.

Ivanovič J., Jankovic-Hladni M. (1991) Hormones and Metabolism in Insect Stress. *Boca Raton, CRC* Press.

Jacobson K.A., Gao Z.G. (2009) Adenosine. In: Malenka R.C. (Ed.), Intercellular Communication in the Nervous System. *Elsevier, Boston, USA*. pp. 627–638.

Jansen A.S., Nguyen X.V., Karpitskiy V., Mettenleiter T.C., Loewy A.D. (1995) Central command neurons of the sympathetic nervous system: basis of the fight-or-flight response. *Science*. 270: 644–46

Jedličková V., Jedlička P., Lee H.J. (2015) Characterization and expression analysis of adipokinetic hormone and its receptor in eusocial aphid *Pseudoregma bambucicola*. *Gen. Comp. Endocrinol.* 223: 38–46.

Jiang H., Vilcinskas A., Kanost M. (2010) "Immunity in Lepidopteran insects," in Invertebrate Immunity, ed K. *Söderhäll (New York, NY: Springer)*. 181–204.

Johansson M.W., Söderhäll K. (1992) Cellular defence and cell adhesion in crustaceans. *Parasitol. Today*. 1: 97-107.

Kaufmann C., Merzendorfer H., Gäde G. (2009) The adipokinetic hormone system in Culicinae (Diptera: Culicidae): molecular identification and characterization of two adipokinetic hormone (AKH) precursors from *Aedes aegypti* and *Culex pipiens* and two putative AKH receptor variants from *A. aegypti*. *Insect Biochem. Mol. Biol. 39*: 770–781.

Kaya H.K., Gaugler R. (1993) *Entomopathogenic nematodes*. *Annu. Rev. Entomol.* 38: 181–206.

Kodrík D. (2008) Adipokinetic hormone functions that are not associated with insect flight. *Physiol. Entomol.* 33: 171–180.

Kodrík D., Bártů I., Socha R. (2010) Adipokinetic hormone (Pyrap-AKH) enhances the effect of a pyrethroid insecticide against the firebug *Pyrrhocoris apterus*. *Pest Manag. Sci*. 66: 425–431.

Kodrík D., Bednářová A., Zemanová M., Krishnan N. (2015a) Hormonal regulation of response to oxidative stress in insects - an update. *Int. J. Mol. Sci.* 16: 25788-25816.

Kodrík D., Krishnan N., Habuštová O. (2007) Is the titer of adipokinetic peptides in *Leptinotarsa decemlineata* fed on genetically modified potatoes increased by oxidative stress? *Peptides*. 28: 974–980.

Kodrík D., Plavšin I, Velki M., Stašková T. (2015b) Enhancement of insecticide efficacy by adipokinetic hormones. In: Montgomery J. (Ed.) *Insecticides: Occurrence, Global Threats and Ecological Impact, Nova Science Publishers, Inc., New York,* eBook, p. 77-91.

Kodrík D., Socha R. (2005) The effect of insecticide on adipokinetic hormone titre in insect body. *Pest Manag. Sci.* 61: 1077–1082.

Kodrík D., Socha R., Syrová Z. (2003) Developmental and diel changes of adipokinetic hormone in CNS and hemolymph of the flightless wing-polymorphic bug, *Pyrrhocoris apterus*. *J. Insect Physiol*. 49: 53–61.

Kodrík D., Vinokurov K., Tomčala A., Socha R. (2012) The effect of adipokinetic hormone on midgut characteristics in *Pyrrhocoris apterus L.* (Heteroptera). *J. Insect. Physiol*. 58: 194-204.

Konuma T., Morooka N., Nagasawa H., Nagata S. (2012). Knockdown of the adipokinetic hormone receptor increases feeding frequency in the two-spotted cricket *Gryllus bimaculatus*. *Endocrinology*. 153: 3111–3122.

Koppenhöfer A.M. (2007) Field manual of techniques in invertebrate pathology: Application and evaluation of pathogens for control of insects and other invertebrate pests. *second ed. Dordrecht: Springer. Nematodes. Pp*. 249–264 in Lacey L.A., Kaya H.K. eds.

Krecek R.C., Waller P.J. (2006) Towards the implementation of the ''basket of options'' approach to helminth parasite control of livestock: emphasis on the tropics/subtropics. *Vet. Parasitol*. 139: 270–282.

Krishnan N., Kodrík D. (2012) Endocrine control of oxidative stress in insects *in:* Farooqui Lamprou I., Mamali I., Dallas K., Fertakis V., Lampropoulou M., Marmaras V.J. (2007) Distinct signalling pathways promote phagocytosis of bacteria, latex beads and lipopolysaccharide in medfly haemocytes. *Immunology*. 121: 314-327.

Krishnan N., Kodrík D., K1udkiewicz B., Sehnal F. (2009) Glutathione–ascorbic acid redox cycle and thioredoxin reductase activity in the digestive tract of *Leptinotarsa decemlineata* (Say). *Insect Biochem. Mol. Biol*. 39: 180 – 188

Kryukova N.A., Chertkova E.A., Semenova A.D., Glazachev Y.I., Slepneva I.A., Glupov V.V. (2015) Venom from the ectoparasitic wasp *Habrobracon hebetor* activates calcium dependent degradation of *Galleria mellonella* larval hemocytes. *Arch. Insect Biochem. Physiol*. 90: 117-130.

Kryukova N.A., Dubovskiy I.M., Chertkova E.A., Vorontsova Y.L., Slepneva I.A., Glupov V.V. (2011) The effect of Habrobracon hebetor venom on the activity of the prophenoloxidase system, the generation of reactive oxygen species and encapsulation in the hemolymph of *Galleria mellonella* larvae. *J. Insect Physiol*. 57: 796–800.

Kryukova N.A., Dubovskiy I.M., Gryzanova E.A., Naumkina V.V., Glupov V.V. (2007) Cell immunity response of the *Galleria mellonella* (L.) (Lepidoptera, Piralidae) during parasitization of *Habrobracon hebetor* (Say) (Hymenoptera, Braconidae). *Euroasian Entomol. J*. 6: 361–364.

Kučerová L., Brož V., Fleischmannová J., Šantrůčková J., Sidorov R., Doležal V., Žurovec M. (2012) Characterization of the *Drosophila* adenosine receptor: the effect of adenosine analogs on cAMP signalling in *Drosophila*

cells and their utility for in vivo experiments. *Journal of Neurochemistry*. 121: 383-395.

Lamprou I., Mamali I., Dallas K., Fertakis V., Lampropoulou M., Marmaras V.J. (2007) Distinct signalling pathways promote phagocytosis of bacteria, latex beads and lipopolysaccharide in medfly haemocytes. *Immunology*. 121: 314-327.

Laughton A.M., Garcia J.R., Altincicek B., Strand M.R., Gerardo N.M. (2011) Characterisation of immune responses in the pea aphid, *Acyrthosiphon pisum*. *J. Insect Physiol.* 57: 830-839.

Lavine M.D., Strand M.R. (2002) Insect hemocytes and their role in immunity. *Insect Biochem Mol Biol.* 32:1295–1309.

Lee G., Park J. H. (2004) Hemolymph sugar homeostasis and starvation-induced hyperactivity affected by genetic manipulations of the adipokinetic hormone- encoding gene in *Drosophila melanogaster*. *Genetics.* 167: 311-323.

Lemaitre B., Hoffmann J. (2007) The host defense of *Drosophila melanogaster*. *Annu Rev Immunol.* 25: 697-743.

Li C., Yun X., Hu X., Zhang Y., Sang M., Liu X., et al. (2013). Identification of G protein-coupled receptors in the pea aphid, *Acyrthosiphon pisum*. *Genomics.* 102: 345–354.

Li X.Y., Cowles R.S., Cowles E.A., Gaugler R., Cox-Foster D.L. (2007) Relationship between the successful infection by entomopathogenic nematodes and the host immune response. *Int J Parasitol.* 37: 365–374.

Litman G.W., Rast J.P., Fugmann S.D. (2010) The origins of vertebrate adaptive immunity. *Nat Rev Immunol.* 10(8):543–53.

Marmaras V.J., Lampropoulou M. (2009) Regulators and signalling in insect hemocyte immunity. *Cell Signal.* 21: 186-195.

Mavrouli M.D., Tsakas S., Theodorou G.L., Lampropoulou M., Marmaras V.J. (2005) MAP kinases mediate phagocytosis and melanization via prophenoloxidase activation in medfly hemocytes. *Biochim. Biophys. Acta.* 1744: 145-156.

Mayack C., Natsopoulou M.E., McMahon D.P. (2015) *Nosema ceranae* alters a highly conserved hormonal stress pathway in honeybees. *Insect Mol Biol.* 24(6): 662-70.

Medzhitov R., Janeway C., Jr. (2000) Innate immunity. *N Engl J Med.* 343(5): 338–44.

Medzhitov R., Janeway C.A. (1997) Innate immunity: impact on the adaptive immune response. *Curr. Opin. Immunol.* 9: 4-9.

Meister M., Lagueux M. (2003) *Drosophila* blood cells. *Cell. Microbiol.* 5: 573-580.

Milde J.J., Ziegler R., Wallstein M. (1995) Adipokinetic hormone stimulates neurons in the insect central nervous system. *J. Exp. Biol.* 198: 1307-1311.

Moshitzky P., Henzel W.J., Rafaeli A., *et al.* (1987a) Synthesis of adipokinetic hormone (AKH-I) in the locust brain. *Insect Biochem.* 17: 1133–1137.

Müller U., Vogel P., Alber G., Schaub G.A. (2008) The innate immune system of mammals and insects. *Contrib Microbiol.* 15: 21–44.

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

Nappi A.J., Vass E. (2001) Cytotoxic reactions associated with insect immunity. *Adv. Exp. Med. Biol.* 484: 329-348.

Nappi A.J., Vass E., Frey F., Carton Y. (1995) Superoxide anion generation in *Drosophila* during melanotic encapsulation of parasites. *Eur. J. Cell Biol.* 68: 450-456,

Němec V., Kodrík D., Matolín S., Laufer H. (1993) Juvenile hormone effects of retinoic acid in insect metamorphosis, embryogenesis and reproduction. *J. Insect Physiol.* 39: 1083-1093.

O'Shea M., Witten J., Schaffer M. (1984) Isolation and characterization of two my active neuropeptides: further evidence for an invertebrate peptide family. *Journal of Neuroscience,* 4: 521-529.

Park H.G., Lee S.K., Kim B.Y., Yoon H.J., Choi Y.S., Lee K.Y., Wan H., Li J., Jin B.R. (2018) Honeybee (*Apis cerana*) vitellogenin acts as antimicrobial and antioxidant agent in the body and venom. *Dev. Comp. Immunol.* 85: 51-60.

Park Y., Kim Y.J., Adams M.E. (2002) Identification of G protein-coupled receptors for *Drosophila* PRXamide peptides, CCAP, corazonin, and AKH supports a theory of ligand-receptor coevolution. *Proc. Natl. Acad. Sci. USA* 99: 11423-11428.

Pennacchio F., Caccia S., Digilio M.C. (2014) Host regulation and nutritional exploitation by parasitic wasps. *Curr. Opin. Insect Sci.* 6: 74-79.

Pennacchio F., Strand M.R. (2006) Evolution of developmental strategies in parasitic hymenoptera. *Annu. Rev. Entomol.* 51: 233–58.

Peters W. (1992) Functions of Peritrophic Membranes. In: Peritrophic Membranes. Zoophysiology, vol 30. *Springer, Berlin, Heidelberg*

Plavšin I., Stašková T., Šerý M., Smýkal V., Hackenberger B.K., Kodrík D. (2015) Hormonal enhancement of insecticide efficacy in *Tribolium*

castaneum: Oxidative stress and metabolic aspects. *Comp. Biochem. Physiol. C.* 170: 19–27.

Pretzel J., Mohring F., Rahlfs S., Becker K. (2013) Antiparasitic peptides. *Adv. Biochem. Eng. Biotechnol.* 135: 157–192.

Ratcliffe N.A., Gagen S.J. (1977) Studies on the in vivo cellular reactions of insects: an ultrastructural analysis of nodule formation in *Galleria mellonella*. *Tissue Cell.* 9:73–85.

Richards A.G., Richards P. A. (1977) The peritrophic membranes of insects. *Annu. Rev. Entomol.* 22: 219-240.

Salmela H., Amdam G.V., Freitak D. (2015) Transfer of immunity from mother to offspring is mediated via egg-yolk protein vitellogenin. *PLoS Pathog.* 11: e1005015.

Salmela H., Sundstrom L. (2017) Vitellogenin in inflammation and immunity in social insects. *Inflamm. Cell Signal.* 4: e1506.

Satyavathi V.V., Minz A., Nagaraju J. (2014) Nodulation: an unexplored cellular defense mechanism in insects. *Cell Signal.* 26: 1753-1763.

Scapagliati G., Mazzini M. (1994) In vivo and in vitro phagocytosis by hemocytes of the stick insect *Bacillus rossius*. *Boll. Zool.* 61: 115-20.

Scarborough R.M., Jamieson G.C., Kalisz F., Kramer S.J., McEnroe G.A., Miller C.A., Schooled D.A. (1984) Isolation and primary structure of two peptides with carclio acceleratory and hyperglycemic activity from the corpora cardiaca of *Periplaneta americana. Proc. Natl. Acad. Sci. USA.* 81: 5575-5579.

Schooneveld H., Romberg-Privee H.M., Veenstra J.A. (1985) Adipokinetic hormone immunoreactive peptide in the endocrine and central nervous system of several insect species: a comparative immunocytochemical approach. *Gen. Comp. Endocrinol.* 57: 184–194.

Scott G.K., Kushner P., Vigne J.L., Benz C.C. (1991) Truncated forms of DNA-binding estrogen receptors in human breast cancer. *J Clin Invest. Aug.* 88(2):700-6.

Shaik H. A., Mishra A., Kodrík D. (2017) Beneficial effect of adipokinetic hormone on neuromuscular paralysis in insect body elicited by braconid wasp venom. *Comp. Biochem. Physiol C.* 196: 11-18.

Shapiro-Ilan D.I., Bruck D.J., Lacey L.A. (2012) Principles of epizootiology and microbial control. Pp. 29–72 in Vega F.E., Kaya H.K. eds. *Insect pathology, second ed. San Diego: Academic Press.*

Shapiro-Ilan D.I., Dawn H.G., Simon J.P., Jane P.F. (2006) Application technology and environmental considerations for use of entomopathogenic nematodes in biological control. *Biol. Control.* 38: 124–133.

Shia A.K., Glittenberg M., Thompson G., Weber A.N., Reichhart J.M., Ligoxygakis P. (2009) Toll-dependent antimicrobial responses in *Drosophila* larval fat body require Spätzle secreted by haemocytes. *J. Cell Sci.* 122: 4505-4515.

Sicard M., Raimond M., Prats O., Lafitte A., Braquart-Varnier C. (2008) Pathogenic effect of entomopathogenic nematode–bacterium complexes on terrestrial isopods. *J Invert. Pathol*. 99: 20–27.

Sideri M., Tsakas S., Markoutsa E., Lampropoulou M., Marmaras V.J. (2008) Innate immunity in insects: surface-associated dopa decarboxylase-dependent pathways regulate phagocytosis, nodulation and melanization in medfly haemocytes. *Immunology*. 123: 528-537.

Silva C.P., Waterfield N.R., Daborn P.J., Dean P., Chilver T., Au C.P., et al. (2002) Bacterial infection of a model insect: *Photorhabdus luminescens* and *Manduca sexta*. *Cell Microbiol*. 4: 329-39.

Singh N.K., Pakkianathan B.C., Kumar M., Prasad T., Kannan M., Konig S. Krishnan M. (2013) Vitellogenin from the silkworm, *Bombyx mori*: an effective anti- bacterial agent. *PLoS One*. 8: e73005.

Sinpoo C., Paxton R.J., Disayathanoowat, T., Krongdang S., Chantawannakul P. (2018) Impact of *Nosema ceranae* and *Nosema apis* on individual worker bees of the two-host species (*Apis cerana* and *Apis mellifera*) and regulation of host immune response. *J. Insect Physiol*. 105: 1-8.

Sláma K., Lukáš J. (2011) Myogenic nature of insect heartbeat and intestinal peristalsis, revealed by neuromuscular paralysis caused by the sting of a braconid wasp. *J. Insect Physiol*. 57: 251-259.

Slavnova T.I., Antonov S.M., Magazanik L.G., Tonkikh A. K., Kosovskii A.V., Sadykov A.A., Abduvakhabov A.A. (1987) *Effect of toxin from the venom of the ichneumon Habrobracon hebetor* (Say) on neuromuscular transmission in insects. *Dok. Akad. Nauk SSSR*. 297: 492-494.

Smith S.M., Vale W.W. (2006) The role of the hypothalamic-pituitary-adrenal axis in neuroendocrine responses to stress. *Dialogues Clin. Neurosci*. 8: 383–395.

Socha R., Kodrík D., Zemek R. (1999) Stimulation of locomotion in *Pyrrhocoris apterus* (Heteroptera: Pyrrhocoridae) is wing-morph independent and correlated with lipid mobilization by adipokinetic hormone. *Naturwissenschaften*. 88: 85-86.

Staubli F., Jorgensen T.J.D., Cazzamal G., Williamson M., Lenz C., Sondergaard L., Roepstorff P., Grimmelikhuijzem C.J.P. (2002) Molecular identification of the insect adipokinetic hormone receptors. *Proc. Natl. Acad. Sci. USA* 99: 3446-3451.

Strand M.R. (2008) The insect cellular immune response. *Insect Sci*. 15: 1-14.

Sturm S., Ramesh D., Brockmann A., Neupert S., Predel R. (2016). Agatoxin-like peptides in the neuroendocrine system of the honey bee and other insects. *J Proteomics*. 132: 77-84.

Tzou P., Ohresser S., Ferrandon D., Capovilla M., Reichhart J. M., Lemaitre B., Hoffmann J. A., Imler J. L. (2000) Tissue-specific inducible expression of antimicrobial peptide genes in *Drosophila* surface epithelia. *Immunity*. 13: 737–748.

Uvell H., Engstrom Y. (2007) A multilayered defense against infection: combinatorial control of insect immune genes. *Trends Genet*. 23: 342–349.

Valko M., Leibfritz D., Moncol J., Cronin M.T., Mazur M., Telser J. (2007) Free radicals and antioxidants in normal physiological functions and human disease. *Int. J. Biochem Cell Biol.* 39(1): 44-84.

Večeřa J., Krishnan N., Alquicer G., Kodrík D., Socha R. (2007) Adipokinetic hormone- induced enhancement of antioxidant capacity of *Pyrrhocoris apterus* hemolymph in response to oxidative stress. *Comp. Biochem. Physiol.* 146: 336–342.

Večeřa J., Krishnan N., Mithöfer A., Vogel H., Kodrík D. (2012) Adipokinetic hormone- induced antioxidant response in *Spodoptera littoralis*. *Comp. Biochem. Physiol.* 155: 389–395.

Veenstra J.A. (2014) The contribution of the genomes of a termite and a locust to our understanding of insect neuropeptides and neurohormones. *Front Physiol*. 5: 454.

Velki M., Kodrík D., Večeřa J., Hackenberger B.K., Socha R. (2011) Oxidative stress elicited by insecticides: A role for the adipokinetic hormone. *Gen. Comp. Endocrinol*. 172: 77–84.

Verlinden H., Vleugels R., Zels S., Dillen S., Lenaerts C., Crabbe K., Spit J., Vanden Broeck J. (2014) Receptors for neuronal or endocrine signalling molecules as potential targets for the control of insect pests. *In: Cohen, E. (Ed.), Advances in Insect Physiology. Elsevier Science Publishers, Londo.*, pp. 167–303.

Wago H. (1991) Phagocytic recognition in *Bombyx mori*. In Immunity of Insects and Other Arthropods, ed. APGupta, pp. 215-35. *Boca Raton: CRC Press Wallingford: CABI Publishing*.

Wang W.X., Wang Y.P., Deng X.J., Dang X.L., Tian J.H., Yi H.Y., Li Y.F., He X.F., Cao Y., Xia Q.Y., et al. (2009) Molecular and functional characterization of a c-type lysozyme from the Asian corn borer, *Ostrinia furnacalis*. *J. Insect Sci.* 9: 17.

Wang Y., Brent C.S., Fennern E., Amdam G.V. (2012) Gustatory perception and fat body energy metabolism are jointly affected by vitellogenin and juvenile hormone in honey bees. *PLoS Genet*. 8(6): e1002779.

Waterfield N.R., Ciche T., Clarke D. (2009) *Photorhabdus* and a host of hosts. *Annu Rev Microbiol*. 63: 557-74.

Whiteside C., Hassan H.M. (1987) Induction and inactivation of catalase and superoxide dismutase of *Escherichia coli* by ozone. *Arch. Biochem. Biophys.* 257: 464–471.

Wicher D., Agricola H.J., Söhler S., Gundel M., Heinemann S.H., Wollweber L., Stengl M., Derst C. (2006) Differential receptor activation by cockroach adipokinetic hormones produces differential effects on ion currents, neuronal activity, and locomotion. *J Neurophysiol*. 95(4): 2314-25.

Wiesner J., Vilcinskas A. (2010) Antimicrobial peptides: the ancient arm of the human immune system. *Virulence* 1: 440–464.

Wigglesworth V.B. (1965) The Principles of Insect Physiology. *London: Methuen.*

Yegutkin G.G. (2008) Nucleotide- and nucleoside-converting ectoenzymes: important modulators of purinergic signalling cascade. *Biochim. Biophys. Acta*. 1783: 673-694.

Yi H.Y., Chowdhury M., Huang Y.D., Yu X.Q. (2014) Insect antimicrobial peptides and their applications. *Appl. Microbiol. Biotechnol*. 98: 5807–5822.

Yokoo S., Goetz P., Tojo S. (1995) Phagocytic activities of hemocytes separated by two simple methods from larvae of two lepidopteran species, *Agrotis segetum* and *Galleria mellonella*. *Appl. Entomol. Zool.* 30: 343-350.

Zasloff M. (2002) Antimicrobial peptides of multicellular organisms. *Nature.* 415: 389-395.

Zemanová M., Stašková T., Kodrík D. (2016) Role of adipokinetic hormone and adenosine in the anti-stress response in Drosophila melanogaster. J. Insect Physiol. 91–92, 39–47.

3. Objectives – The aim of the study

The main aim of this thesis was to investigate interactions between the infection elicited by entomopathogenic, and adipokinetic hormones or vitellogenin. Possible similarities between AKH and adenosine in the antistress reaction were studied as well. For those various markers were monitored: mortality, total metabolism, level of nutrients, level of AKH in CNS and haemolymph, and anti-oxidative stress response. Comparison of the nematodal and fungal infections was performed as well.

4. Results and conclusions

The results of this thesis are included in two published papers and in one submitted manuscript.

Paper 1: Ibrahim E., Hejníková M., Shaik H.A., Doležel D., Kodrík, D. (2017) Adipokinetic
hormone activities in insect body infected by entomopathogenic nematode. *J. Insect*
Physiol. 98: 347–355.

This paper describes an AKH role in antistress reaction elicited by the entomopathogenic nematode *Steinernema carpocapsae* infection in the firebug *Pyrrhocoris apterus* adults.

The role of adipokinetic hormone (AKH) in the firebug *Pyrrhocoris apterus* adults infected by the entomopathogenic nematode (EPN) *Steinernema carpocapsae* was examined in this study. It was found that co-application of EPN and AKH enhanced firebug mortality about 2.5 times within 24 h (from 20 to 51% in EPN vs. EPN + AKH treatments), and resulted in metabolism intensification, as carbon dioxide production in firebugs increased about 2.1 and 1.6 times compared to control- and EPN-treated insects, respectively. Accordingly, firebugs with reduced expression of AKH receptors showed significantly lower mortality (by 1.6 to 2.9-folds), and lower general metabolism after EPN + AKH treatments. In addition, EPN application increased Akh gene expression in the corpora cardiaca (1.6 times), AKH level in the corpora cardiaca (1.3 times) and hemolymph (1.7 times), and lipid and carbohydrate amounts in the haemolymph. Thus, the outcomes of the present study demonstrate the involvement of AKH into the antistress reaction elicited by the nematobacterial infection. The exact mechanism by which AKH acts is unknown, but results suggested that the increase of metabolism and nutrient amounts in hemolymph might play a role.

Paper 2: Ibrahim E., Dobeš P., Kunc M., Hyršl P., Kodrík D. (2018) Adipokinetic hormone and adenosine interfere with nematobacterial infection and locomotion in *Drosophila melanogaster*. *J. Insect Physiol.* 107: 167–174.

The role of AKH and adenosine in defense reaction against the nematode *Steinernema carpocapsae*, and the effect of AKH and adenosine receptor deficiency on *Drosophila* locomotion was studied in this paper.

This study examined how adipokinetic hormone (AKH) and adenosine affect defense responses in *Drosophila melanogaster* larvae infected with entomopathogenic nematodes (EPN, *Steinernema carpocapsae* and *Heterorhabditis bacteriophora*). Three loss-of-function mutant larvae were tested: AKH^1, $AdoR^1$ (adenosine receptor), and AKH^1 $AdoR^1$. Mortality decreased in all mutants post-EPN infection compared with the control (w^{1118}). Additionally, co-application of external AKH with EPN significantly increased mortality beyond rates observed in EPN-only treatment, while also elevating carbon dioxide production, a measure of metabolism. Furthermore, trehalose levels increased in both w^{1118} and AKH^1 larvae post-EPN infection, but the latter group exhibited a lower increase and total trehalose levels. Interestingly, baseline trehalose was relatively high in untreated $AdoR^1$ and AKH^1 $AdoR^1$ mutants, with levels remaining unaffected by infection. Infection also elevated hemolymph lipid content overall, but the different mutations did not substantially influence this change. In contrast, hemolymph protein content dropped after EPN infection in all tested groups, but this decline was more intense among AKH^1. In uninfected larvae mutations decreased anti-oxidative capacity in AKH^1 and increased in AdoR1, however, its post-infection increases were similar in all mutants, suggesting that antioxidant response in *Drosophila* involves mechanisms also beyond AKH and adenosine. Furthermore, AKH application in w^{1118} larvae significantly increased movement distance and percentage of larval activity, but reduced velocity. Mutations of AKH and AdoR did not strongly affect locomotion.

Paper 3: Kodrík D., Ibrahim E., Gautam U. K., Frydrychová R., Bednářová A., Krištůfek V., and Jedlička P. Changes in vitellogenin expression caused by nematodal and fungal infections in insects. *J. Exp. Biol.* (submitted for publication).

The role of vitellogenin in defense reaction against nematodal (*Steinernema carpocansae*) and fungal (*Isaria fumosorosea*) infections was examined in this study.

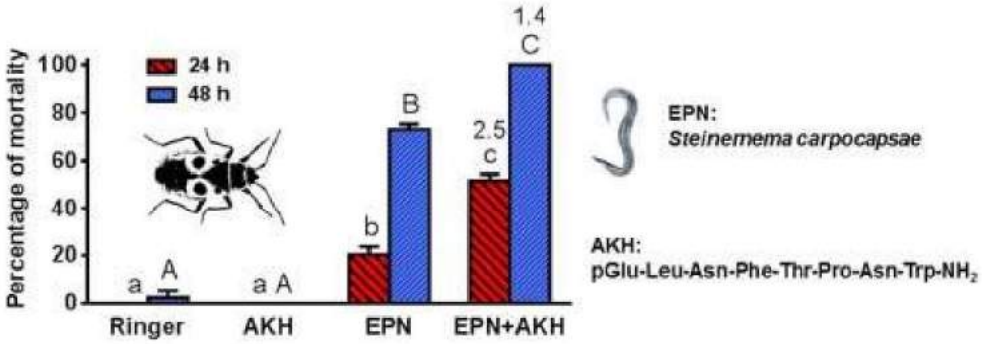
This study examined the expression and role of vitellogenin (Vg) in the body of the firebug
Pyrrhocoris apterus (Heteroptera, Insecta) during the infection elicited by two entomopathogenic organisms, the nematode *Steinernema carpocapsae* and the fungus *Isaria fumosorosea*. Infection by *S. carpocapsae*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

In conclusion, the results of this thesis demonstrate the involvement of AKH into the antistress reaction elicited by the nematobacterial infection in *Pyrrhocoris apterus* and *Drosophila melanogaster*. On the other hand, the role of adenosine in *Drosophila* model is smaller and somewhat unclear. Further, the results from the last part of this study demonstrate an important role of Vg protein in defense response against nematodal and fungal infections in firebugs' body. Taken together, we believe that all these findings are academically interesting but also contribute results that might be potentially useful in pest management strategies in future: any natural agent able to intensify toxicity of both entomopathogenic organisms and classical insecticides would be undoubtedly interesting for the insect pest control and protection of the environment.

5. Papers

5.1. Paper 1

Ibrahim E., Hejníková M., Shaik H. A., Doležel D., Kodrík D. (2017) Adipokinetic hormone activities in insect body infected by entomopathogenic nematode. *J. Insect Physiol*. 98: 347-355.



Journal of Insect Physiology 98 (2017) 347–355

Contents lists available at ScienceDirect

Journal of Insect Physiology

journal homepage: www.elsevier.com/locate/jinsphys

Adipokinetic hormone activities in insect body infected by entomopathogenic nematode

Emad Ibrahim [a,b,c], Markéta Hejníková [a,b], Haq Abdul Shaik [a], David Doležel [a], Dalibor Kodrík [a,b,*]

[a] Institute of Entomology, Biology Centre, CAS, Branišovská 31, 370 05 České Budějovice, Czech Republic
[b] Faculty of Science, University of South Bohemia, Branišovská 31, 370 05 České Budějovice, Czech Republic
[c] Faculty of Agriculture, University of Cairo, Giza, Egypt

ARTICLE INFO

Article history:
Received 5 January 2017
Received in revised form 22 February 2017
Accepted 25 February 2017
Available online 27 February 2017

Keywords:
Mortality
Akh gene expression
AKH receptor
Metabolism
RNAi
Pyrrhocoris apterus

ABSTRACT

The role of adipokinetic hormone (AKH) in the firebug *Pyrrhocoris apterus* adults infected by the entomopathogenic nematode (EPN) *Steinernema carpocapsae* was examined in this study. It was found that co-application of EPN and AKH enhanced firebug mortality about 2.5 times within 24 h (from 20 to 51% in EPN vs. EPN + AKH treatments), and resulted in metabolism intensification, as carbon dioxide production in firebugs increased about 2.1 and 1.6 times compared to control- and EPN-treated insects, respectively. Accordingly, firebugs with reduced expression of AKH receptors showed a significantly lower mortality (by 1.6 to 2.9-folds), and lower general metabolism after EPN + AKH treatments. In addition, EPN application increased *Akh* gene expression in the corpora cardiaca (1.6 times), AKH level in the corpora cardiaca (1.3 times) and haemolymph (1.7 times), and lipid and carbohydrate amounts in the haemolymph. Thus, the outcomes of the present study demonstrate involvement of AKH into the anti-stress reaction elicited by the nematobacterial infection. The exact mechanism by which AKH acts is unknown, but results suggested that the increase of metabolism and nutrient amounts in haemolymph might play a role.

1. Introduction

Entomopathogenic nematodes (EPNs) are obligate parasites that complete their entire life cycle in insect hosts, causing diseases and ultimately killing their hosts (Shapiro-Ilan et al., 2006). Like many other nematode parasites, EPNs are symbiotic with bacteria that help them kill the host and use their tissues to produce nutrients for the new EPN generation. These symbiotic bacteria are particularly found in the gut of infective juvenile nematodes that are able to attack and invade insects (Ciche and Ensign, 2003). One of the best-known groups of EPNs is that of the species belonging to family *Steinernematidae* (Kaya and Gaugler, 1993; Grewal et al., 2005; Koppenhöfer, 2007). This family carries symbiotic *Xenorhabdus* spp. bacteria that are also insect pathogens. The mutualistic relationship between nematodes and their bacteria is not obligate, as nematodes can kill the host in the absence of their bacteria (Herbert and Goodrich-Blair, 2007; Waterfield et al., 2009).

Insect hosts' defence against EPN invasion relies on their powerful immune responses at the cellular (e.g., phagocytosis, encapsulation) and humoral (e.g., inducible anti-microbial peptides, lysozymes, lectins, prophenoloxidase system) levels to eliminate or reduce the infection (Castillo et al., 2011). These responses contribute locally, systemically and jointly to protect insects against attacks by foreign microorganism (Uvell and Engstrom, 2007).

Although the effects caused by parasitoids on the hormone development of their insect hosts have been described in numerous papers (e.g., review articles Beckage, 1985; Beckage and Gelman, 2001), the interactions between the EPN infection and insect (neuro)hormonal system are still unknown. Therefore, we decided to study the role of adipokinetic hormones (AKHs) in the insect model species the firebug *Pyrrhocoris apterus* (L.) under the stress conditions elicited by EPN infection. AKHs are good candidates for mediating the hormonally controlled defence system responding to EPN infection. They belong to the AKH/RPCH (adipokinetic hormone/red pigment concentrating hormone) peptide family, and are synthesized, stored, and released by neurosecretory cells in the corpora cardiaca, a neuroendocrine gland connected with the brain. AKHs comprise eight to ten amino acids (Gäde et al., 1997) and their signal transduction at the cellular level is well documented for the fat body (see Gäde and Auerswald,

* Corresponding author at: Institute of Entomology, Biology Centre, CAS, Branišovská 31, 370 05 České Budějovice, Czech Republic.
E-mail address: kodrik@entu.cas.cz (D. Kodrík).

http://dx.doi.org/10.1016/j.jinsphys.2017.02.009
0022-1910/

2003). AKH functions resemble those of mammalian glucagon (Bednářová et al., 2013a): these peptides behave as typical stress hormones by stimulating catabolic reactions, mobilising lipids, carbohydrates, and proline to provide energy (Gäde et al., 1997). However, AKHs are pleiotropic, with a number of actions that boost their main roles in energy metabolism (Kodrík, 2008). Among other functions, these peptides stimulate heart beat (Scarborough et al., 1984) and general locomotion (Kodrík et al., 2000), regulate starvation-induced foraging behaviour in *Drosophila* sp. (Lee and Park, 2004), participate in the activation of antioxidant mechanisms (Kodrík et al., 2007, 2015a), enhance food intake and digestive processes in insect gut (Kodrík et al., 2012; Bil et al., 2014; Bodláková et al., 2017), and interact with the humoral and cellular immune systems (Goldsworthy et al., 2002a). All functions at the cellular level are mediated by specific membrane-bound AKH receptors, which are related to the vertebrate gonadotropin-releasing hormone receptors and have been characterized in several insect species, including *Drosophila melanogaster* and *Bombyx mori* (Staubli et al., 2002; Park et al., 2002; Wicher et al., 2006). The AKH receptors are also linked to a G-protein involved in either adenylate cyclase or phospholipase C pathways (Gäde and Auerswald, 2003), or in both pathways (Bednářová et al., 2013b).

There is an intensive effort to utilize insect neurohormones as biorational pesticides in order to reduce the amount of chemical insecticides, to increase protection efficacy in plants and stored products, and to protect the environment (Borovsky and Nauen, 2007; Gäde and Goldsworthy, 2003; Verlinden et al., 2014). Apparently, AKHs or some of their physiological and biochemical functions might also be suitable as biorational pesticides, as AKHs are able to penetrate insects' cuticle (e.g., Kodrík et al., 2002a; Lorenz et al., 2004). Furthermore, recent studies revealed that the co-application of AKH and insecticides, topically or by injection, enhanced their efficacy in the insect body (Kodrík et al., 2010; Velki et al., 2011; Plavšin et al., 2015; reviewed by Kodrík et al., 2015b). Although the mechanism of this synergistic action is unknown, it is hypothesized that AKHs might intensify insecticide action by accelerating metabolite exchange rates, which has been documented by both increased carbon dioxide production, and the penetration of insecticides into tissues (Kodrík et al., 2015b).

The firebug *P. apterus* is an insect model organism in which AKHs have been intensively studied (Kodrík, 2008; Kodrík et al., 2015a). Its two AKHs, Pyrap-AKH (pGlu-Leu-Asn-Phe-Thr-Pro-Asn-Trp-NH_2; Kodrík et al., 2000) and Peram-CAH-II (pGlu-Leu-Thr-Phe-Thr-Pro-Asn-Trp-NH_2; Kodrík et al., 2002b), are well characterised, and the cDNA sequences encoding them are known, together with the amino acid composition of their pre-prohormones (Kodrík et al., 2015c). Therefore, *P. apterus* is an excellent model to explore the role of AKH signalling in defence mechanisms.

The main goal of the present study was explore the putative role of AKH in the processes elicited by EPN infection, and characterize the impact of the infection in insect physiology. The effects that the external application of AKH might have on EPNs infection, and the hormones influencing it, were also studied.

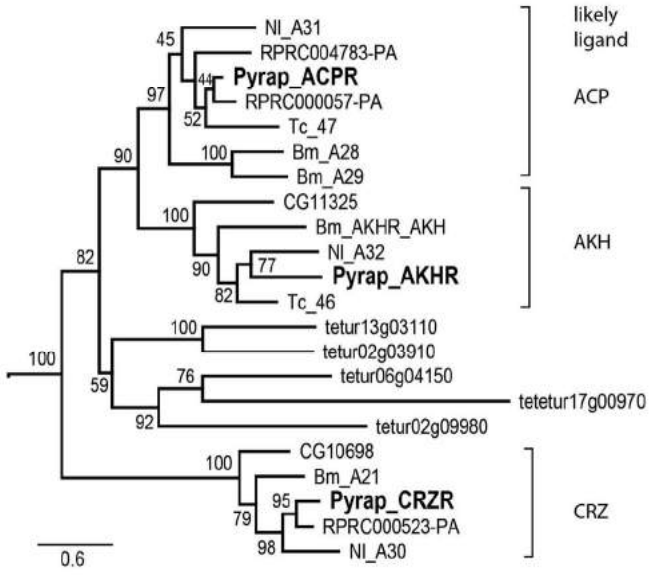


Fig. 1. *P. apterus* contains clear homologs of adipokinetic hormone receptor (Pyrap-AKHR), corazonin receptor (Pyrap-CRZR) and AKH/corazonin-related peptide receptor (Pyrap-ACPR). Phylogenetic tree was obtained from RAxML 7.2.8 analysis of protein sequences under LG + Gamma substitutional model. Bootstrap support from 500 replicates is shown in% under each node. Receptors from *Drosophila* (CG), *Nillaparvata* (NI_), *Tribolium* (Tc_), *Bombyx* (Bm_), *Rhodnius* (RPRC) and *Tetranychus* (tetur) were used as a reference.

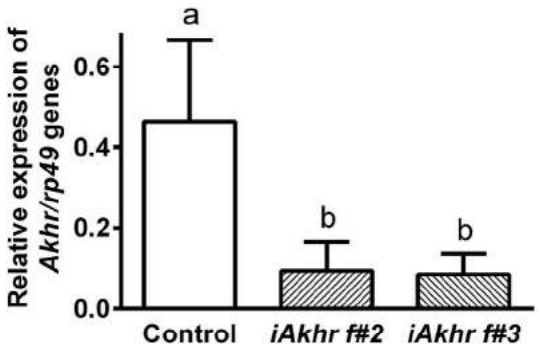


Fig. 2. RNAi mediated knockdown of *Akhr f#2* and *Akhr f#3* gene expression. All data were normalised using expression of *rp49* gene. Statistically significant differences among the groups at the 5% level evaluated by one-way ANOVA with the Tukey's post-test are indicated by different letters; bars = mean ± SD, n = 4–6.

2. Materials and methods

2.1. Experimental insects

A stock culture of the firebug, *P. apterus* (L.) (Heteroptera, Insecta), established from wild populations collected at České Budějovice (Czech Republic, 49° N), was used in the present study. Larvae and adults of a common reproductive brachypterous morph were kept in 0.5 L glass jars in a mass culture (approximately 40 specimens per jar) and reared at constant temperature of 26 ± 1 °C under long-day conditions (18 h light: 6 h dark). They were supplied with linden seeds and water ad libitum, which were replenished twice a week. Freshly ecdysed adults were transferred to small 0.25 L glass jars (females and males separately) and kept under the same photoperiod, food and temperature regimes in which they developed. To work with maximally uniform animals and minimize influence of complex physiology (i.e. female's ovarian cycle), only 7-day old males were used for the experiments.

2.2. Entomopathogenic nematode Steinernema carpocapsae

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

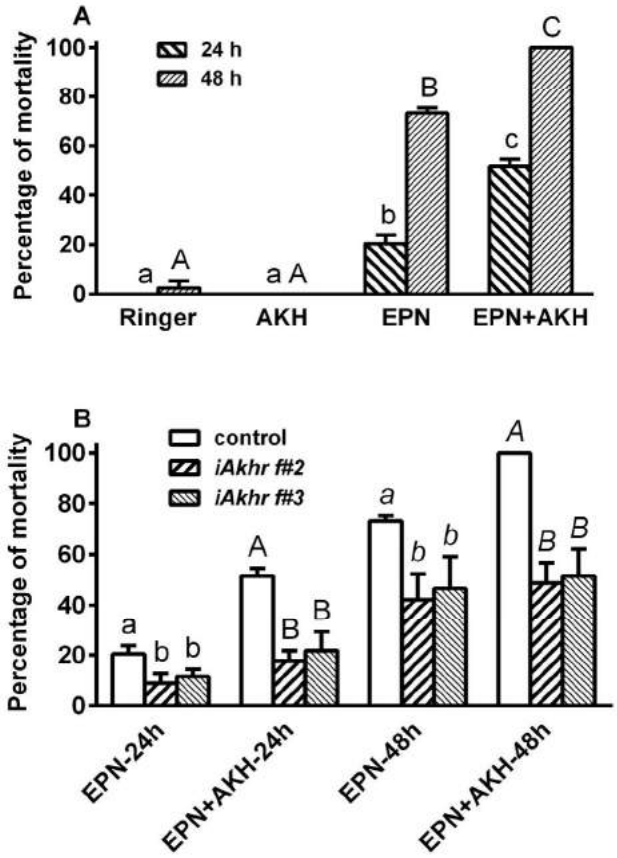


Fig. 3. (A) The effect of entomopathogenic nematode *S. carpocapsae* (EPN; 100 ind./insect) and Pyrap-AKH treatment (AKH; 10 pmol) on mortality of *P. apterus* adults (with normal *Akhr* expression) 24 or 48 h after the treatment. (B) The same effect on *P. apterus* adults with reduced *Akhr* expression (controls = *P. apterus* adults with normal *Akhr* expression). Statistically significant differences among the treatments at the 5% level evaluated by one-way ANOVA with the Tukey's post-test are indicated by different letters; bars = mean ± SD, n = 4–5 groups with 20 individuals in each.

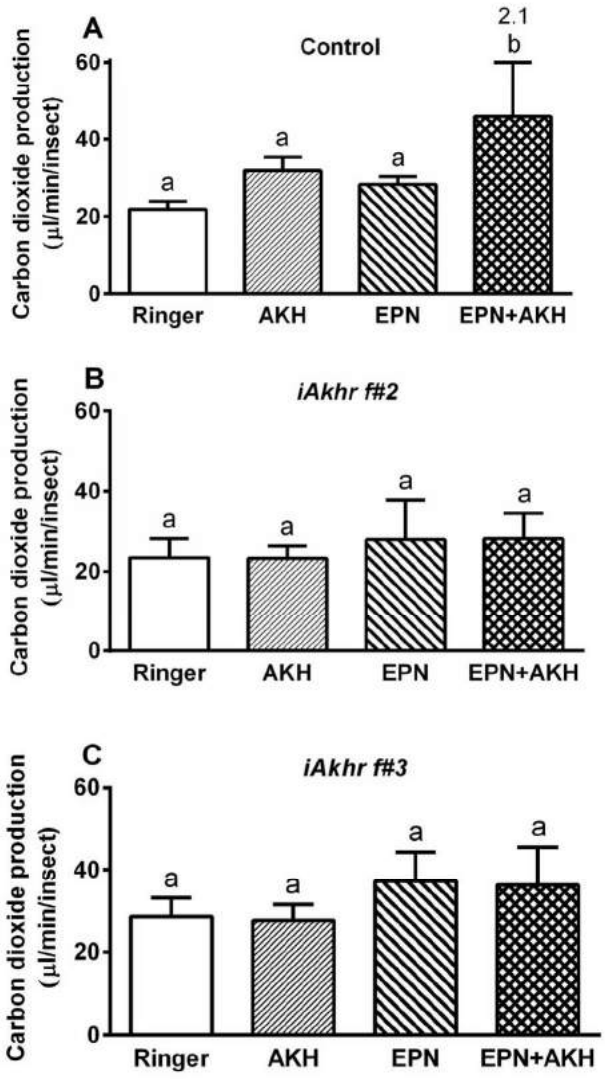


Fig. 4. (A) The effect of entomopathogenic nematode *S. carpocapsae* (EPN; 100 ind./insect) and Pyrap-AKH treatment (AKH; 10 pmol) on carbon dioxide production in *P. apterus* (control) adults 24 h after the nematode and/or 90 min after the Pyrap-AKH treatments. The number above the column represents fold-difference of CO_2 production in the corresponding group as compared with Ringer saline treated individuals. (B, C) The same effect on *P. apterus* adults with reduced *Akhr* expression. 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 = 6–7.

2.3. Nematode and hormone treatments

Twenty *P. apterus* males were transferred in 200 ml glass jar (5.5 cm dimeter, 12.5 cm height) containing one layer of tissue paper soaked with the nematode suspension (100 individuals per bug) and kept under long-day conditions of 26 ± 1 °C. The effect of nematode treatment on studied characteristics (see below) was assayed in males 24 h post infection.

To determine mortality, five groups (each consisting of 20 males) for each experimental treatment were inspected 24 h and 48 h post infection.

Two *P. apterus* adipokinetic hormones, Pyrap-AKH (Kodrík et al., 2000) and Peram-CAH-II (Kodrík et al., 2002b), commercially synthesized by Dr. L. Lepša from Vidia Company (Praha, Czech Republic) were used in this study. In some experiments a dose of 10 pmol Pyrap-AKH (for details of the selected dose, see Kodrík et al., 2000) dissolved in 2 µl 20% methanol in Ringer saline was injected through the metathoracic-abdominal intersegmental membrane into the thorax of the experimental firebugs kept in standard conditions (see Section 2.1); control bugs were injected with 2 µl of solvent only.

2.4. Metabolic rate measuring

A flow-through respirometry system was used to measure a rate of carbon dioxide production of experimental firebugs. Air is pushed through a chamber with the analysed insects in this system at a flow rate 80 ml min^{-1} into the LI-7000 CO_2/H_20 analyser (LI-COR Biosciences, Lincoln, NE, USA), which is interfaced with a computer (for details see Kodrík et al., 2010). The individual bugs were examined 24 h after the *S. carpocapsae* and/or 90 min after the Pyrap-AKH treatments (significant peak of mobilization of metabolites occurs 90 min after the AKH injection – see Kodrík et al., 2002a) in 8 ml chambers (8 chambers were examined at a time) for a period of 40 min; only living individuals were used for the analysis. Data were analysed by the data-acquisition software (Sable Systems, Las Vegas, Nevada, USA). The carbon dioxide production (V_{CO2}) was calculated from fractional concentrations of carbon dioxide going in (FI) and coming out (FE) of the respirometry chamber using a formula according to Withers (1977) and expressed in µl min^{-1} bug^{-1} units:

$$V_{CO2} = (FE_{CO2} - FI_{CO2})\, f$$

where f is the flow rate in µl min^{-1}.

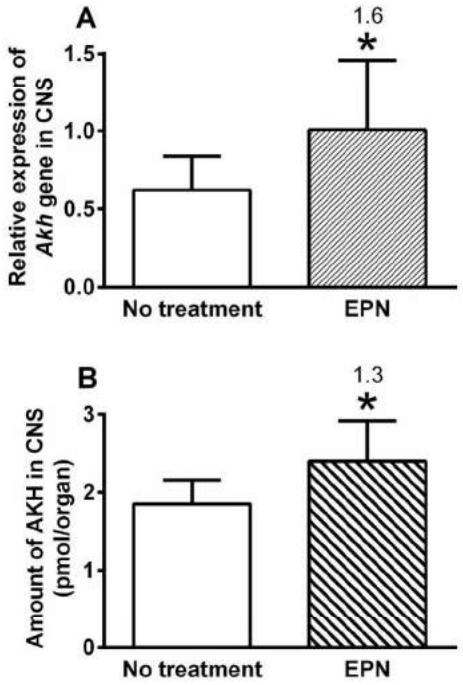


Fig. 5. The effect of entomopathogenic nematode *S. carpocapsae* (EPN; 100 ind./insect) treatment on (A) *Pyrap-Akh* gene expression, and (B) on total AKH amount in *P. apterus* adult CNS 24 h after the treatment. Statistically significant differences between the experimental groups and untreated controls at the 5% level evaluated by Student's *t*-test are indicated by asterisks. The numbers above the columns represent fold-differences of *Pyrap-Akh* gene expression (A) and AKH level (B) in the EPN groups as compared with untreated controls; bars = mean ± SD, n = 10–16.

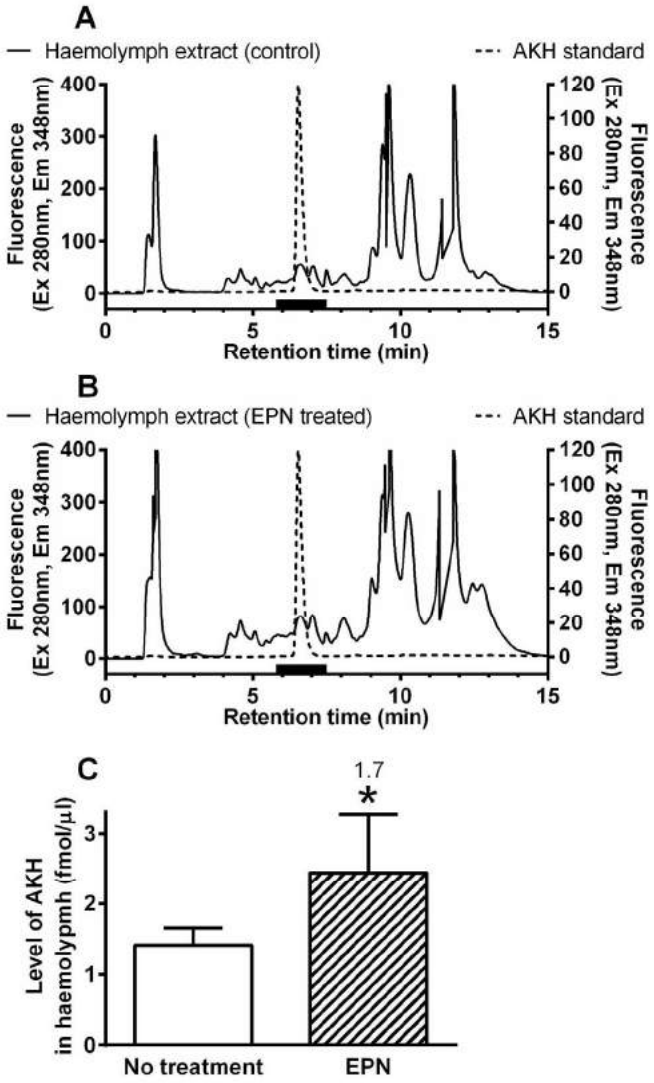


Fig. 6. The RP HPLC elution profiles of an prepurified extract of (A) 125 µl haemolymph from (untreated) *P. apterus* adults, and (B) 175 µl of haemolymph from adults 24 h after the treatment by *S. carpocapsae* (EPN; 100 ind./insect) (solid lines), and profiles of a mixture of AKH standards (Pyrap-AKH – 100 pmol; Peram-CAH-II – 100 pmol) (dashed lines; joint retention time 6.53 min). The ELISA determined level of AKHs (C) in HPLC pre-purified haemolymph (in the fractions marked by the thick horizontal lines in A and B) from *P. apterus* adults. Statistically significant difference between the EPN and control groups at the 5% level evaluated by Student's *t*-test is indicated by asterisk. The number above the column represents fold-difference of AKH level in the EPN group as compared with untreated control; bars = mean ± SD, n = 5–7.

2.5. AKHR sequence and phylogenetic analysis

BLAST-P search of *P. apterus* transcriptome was used to identify AKHR candidates. Top ~25/50 hits where compared in phylogenetic analysis. Clear homologs of AKHR (GenBank acc. number KY110360), CRZR - corazonin receptor - (GenBank acc. number KY110361), and ACPR - AKH/corazonin-related peptide receptor - (GenBank acc. number KY110362) were aligned as protein sequences with corresponding receptors from *Drosophila melanogaster*, *Tribolium castaneum*, *Bombyx mori*, *Tetranychus urticae* (Veenstra et al., 2012), *Nillaparvata lugens* (Tanaka et al., 2014) and *Rhodnius prolixus* (Ons et al., 2016) using MAFFT algorithm (Geneious, Biometers). The alignment was controlled by eye and ambiguously aligned regions were removed. The phylogenetic tree was constructed with RAxML (LG model) in Geneious (Biometers). Bootstrap support was retrieved form 500 replicates. The sequence of AKHR open reading frame was confirmed by PCR and Sanger sequencing.

2.6. Akhr RNAi

Expression of *Akhr* was knocked down by RNA mediated interference (RNAi) using a well-established RNAi approach (Bajgar et al., 2013). To minimize off targeting, two non-overlapping fragments knocking down *Akhr* expression were used independently. First, *Akhr fr#2* (446 bp) and *Akhr fr#3* (479 bp) were PCR-amplified and cloned into pGEM-Teasy (Promega) and verified by Sanger sequencing (see Tables S1 and S2 for primer sequences). The inserts were PCR amplified with T7 primer and pGEM-RNAi, a primer modifying SP6 sequence to T7 sequence (Urbanová et al., 2016, Table S2) and double-strand RNA (dsRNA) was synthesized using T7 MEGAscript kit (Ambion) according to manufacturer's instructions. dsRNA fragments were purified by phenol-chloroform and diluted in a Ringer saline to final concentrations 2 µg/µl. Two µl of dsRNA solutions were injected into two-day after adult ecdysis *P. apterus*. Ringer saline served as a negative control. Injected bugs were supplied with water only for following 24 h, and thereafter kept with linden seeds and water for following 6 days. Sacrificed animals were snap frozen, total RNA was isolated from the whole body, and the efficiency of RNAi knock down was assessed from q-RT-PCR with *Akhr*-specific primers with *rp49* serving as an internal control (see Table S3 for primer sequences and 2.7 for the methodology).

2.7. Quantification of Pyrap-Akh gene expression and Akhr RNAi efficiency

Total RNA was isolated from the CNS (brain with corpora cardiaca) using RiboZol™ RNA Extraction Reagents (AMRESCO, LLC.

Solon, Ohio, USA) following the manufacturer's protocol. RNA isolates were treated with TURBO DNA-free™ DNase (AMBION® by Life Technologies™, Carlsbad, California, USA) to remove traces of contaminant DNA. Reverse transcription was carried out using the Superscript First-Strand Synthesis System for RT-PCR (Invitrogen) on 1 μg of total RNA with random hexamers. Relative transcript levels were measured by quantitative PCR using the iQ SYBR Green Supermix kit and the C1000 Thermal Cycler (both Bio-Rad). All measured transcripts were normalized to relative levels of the ribosomal protein (*rp49*) mRNA as described previously (Doležel et al., 2007). Sequences of primers are listed in Table S3.

2.8. AKH extraction from CNS and haemolymph

Central nervous system (CNS) containing the brain with corpora cardiaca and corpus allatum attached was dissected from the firebug head cut off from the body under the Ringer saline. The AKHs were extracted from the CNS using 80% methanol, the solution was evaporated in a vacuum centrifuge and the resulting pellet stored at −20 °C until needed.

For determination of the endogenous AKH titre in the haemolymph by competitive ELISA (see Section 2.9), some pre-purification steps described in our previous paper (Goldsworthy et al., 2002b) were essential. Briefly, haemolymph samples collected from several dozens of firebugs by cutting off their antennae (volumes 125 resp. 175 μl - see Fig. 6) were extracted in 80% methanol and after centrifugation the supernatants were evaporated to dryness. Then the pellets were dissolved in 0.11% trifluoroacetic acid, applied to a solid phase extraction cartridge Sep Pak C18 (Waters), and eluted by 60% acetonitrile. The eluent was analysed on a Waters HPLC system with a fluorescence detector Waters 2475 (wave length λ_{Ex} – 280 nm; λ_{Em} – 348 nm) using a Chromolith Performance RP-18e column (Merck), solutions A and B (A – 0.11% trifluoroacetic acid in water; B – 0.1% trifluoroacetic acid in 60% acetonitrile) and a flow rate 2 ml/min. Fractions eluting between 5.8 and 7.5 min were subjected to competitive ELISA. Retention times of the two *Pyrrhocoris* synthetic adipokinetic peptides Pyrap-AKH and Peram-CAH-II were identical under the used conditions - 6.53 min.

2.9. ELISA determination of AKH level

A competitive ELISA was used for determination of total AKH content in *P. apterus* CNS (antibody dilution 1:5000, 0.5 CNS equiv. per well, detection limit 20 fmol per well) and haemolymph (antibody dilution 1:1000, 25 μl haemolymph equiv. per well, detection limit 14 fmol per well (unpublished data)) according to our protocol published earlier (Goldsworthy et al., 2002b).

Briefly, rabbit antibodies were raised commercially against Cys^1-Pyrap-AKH (Sigma Genosys, Cambridge, UK) and the resulting antibody recognised well both the Pyrap-AKH and the Peram-CAH-II. A biotinylated probe was prepared from Cys^1-Pyrap-AKH using Biotin Long Arm Maleimide (BLAM, Vector Laboratories, Peterborough, UK). The ELISA comprised pre-coating of the 96-well microtiter plates (high binding Costar, Corning Incorporated, Corning, NY, USA) overnight with the antibody preparation in coating buffer. After blocking (with non-fat dried milk), test samples 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 PBS-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 Pyrap-AKH, which allowed the construction of a competition curve and estimation of the AKH content of unknown samples.

2.10. Spectrophotometric determination of nutrients

The level of lipids and carbohydrates was determined in the firebug haemolymph 24 h after the *S. carpocapsae* treatment. To do that the haemolymph samples were obtained from cutting end of the antenna. Further, haemocytes were removed from the samples by centrifuging at 13,000*g* for 2 min at 4 °C and 1 μl of

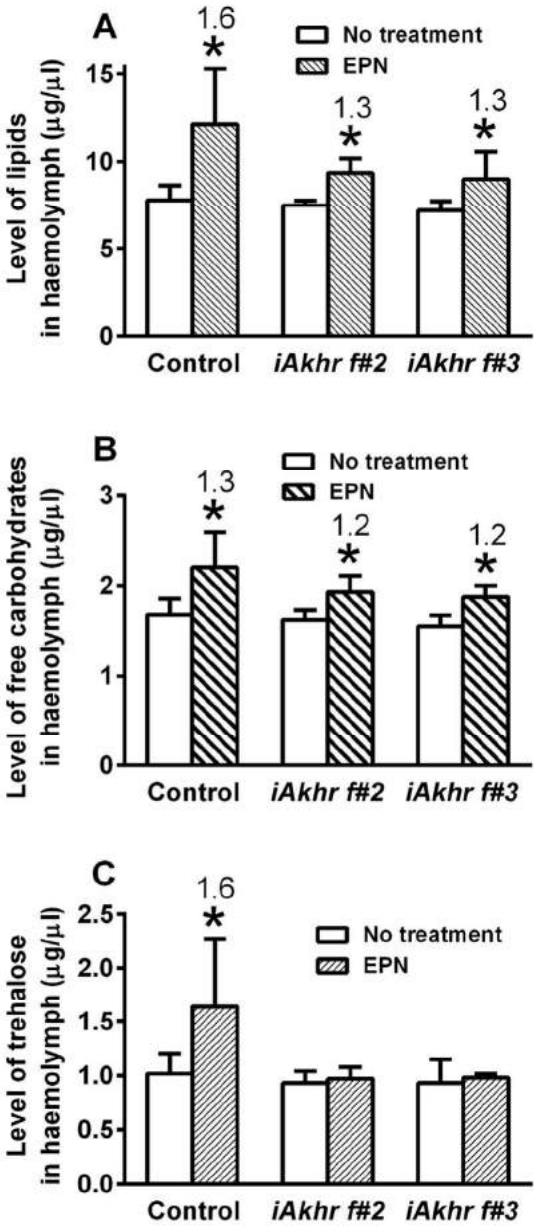


Fig. 7. The effect of entomopathogenic nematode *S. carpocapsae* (EPN; 100 ind./insect) treatment on lipid (A), free carbohydrate (B) and trehalose (C) levels in *P. apterus* adult haemolymph 24 h after the treatment. Both control firebugs and those with reduced *Akhr* expression were used for the experiments. Statistically significant differences between the experimental groups and controls at the 5% level evaluated by Student's *t*-test are indicated by asterisks. The numbers above the columns represents fold-difference of level in the EPN groups as compared with untreated controls; bars = mean ± SD, n = 5.

supernatant per sample was used for determination of the nutrients.

- *Lipid determination* – was done by sulpho-phosho-vanillin method according to Zöllner and Kirsch (1962), as modified for *Pyrrhocoris* 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 (1 μl) was diluted in 39 μl of distilled water and then used for quantification of free carbohydrate level by the anthrone method (Carroll et al., 1956) that was modified for *Pyrrhocoris* by Socha et al. (2004).
- *Trehalose determination* – for trehalose quantification, the reducing sugars were removed from the samples, and then trehalose itself was determined using the Trehalose assay kit, (Megazyme) according to the manufacturer's instructions. Briefly, 10 μl samples (1 μl haemolymph supernatant and 9 μl water) were mixed with an equivalent volume of alkaline borohydrate solution (10 mg/ml sodium borohydrate in 50 mM sodium hydroxide) and incubated for 30 min at 40 °C. Then, the excess of borohydride was removed with 25 μl of 200 mM acetic acid; 5 min later, 10 μl of 2 M imidazole buffer (pH 7.0) was added to adjust the pH level in the sample to the neutral. The trehalose level was determined in 27.5 μl samples using the anthrone reagent as mentioned above for quantification of free carbohydrates.

2.11. 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 bar graphs represent mean ± SD, the numbers of replicates (n) are depicted in the figure legends. The statistical differences were evaluated by Student's *t*-test (Figs. 5–7) and one-way ANOVA with the Tukey's post-test (Figs. 2–4) using the Prism software.

3. Results

3.1. AKH receptors in P. apterus

Because each insect species has numerous neurohormone receptors, often similar in sequence, the unambiguous identification of AKH receptor homolog in *P. apterus* was crucial. The phylogenetic analysis identified four clusters related to AKHR, three of which were supported by high (>97) bootstrap values (Fig. 1). One cluster consisted of corazonin receptors (CRZR), a single Pyrap-CRZR, and one *R. prolixus* sequence; the second cluster comprised five *T. urticae* sequences with unclear ligands; the third cluster grouped AKH receptors (AKHR), one Pyrap-AKHR, and one *N. lugens* sequence, whereas the corresponding AKHR in *R. prolixus* was missing; and the fourth cluster included AKH/corazonin-related peptide receptors (ACPR) with one single Pyrap-ACPR.

3.2. RNAi mediated knockdown

To exclude off-target effects, two non-overlapping dsRNA fragments were used to knockdown the expression of *Pyrap-Akhr*. Injection of dsRNA fragment #2 (*iAkhr fr#2*) or fragment #3 (*iAkhr fr#3*) resulted in a significant and similar efficient knockdown of *Akhr* mRNA, which was approximately 20% of normal expression level (Fig. 2).

3.3. The effects of EPNs and AKH on mortality and metabolism

The impact of Pyrap-AKH on the mortality rates due to EPNs infecting *P. apterus* adults was evaluated. Initial tests revealed that a dose of 100 *S. carpocapsae* individuals per *P. apterus* adult was appropriate for the experiments. This dose elicited a mortality rate around 20% within 24 h and above 70% within 48 h (Fig. 3A); lower or higher doses were not suitable for the experiments as they caused too low or too high mortalities, respectively. Mortalities in the Ringer-treated control group and in the Pyrap-AKH-treated experimental group were negligible or null (Fig. 3A), but the co-application of EPNs and 10 pmol Pyrap-AKH injections increased mortality by 2.5-folds (compared with EPNs alone), approximately (from 20% to 51%) within 24 h; within 48 h, all experimental firebugs treated with EPNs + Pyrap-AKH died (mortality = 100%). Therefore, 100 *S. carpocapsae* per *P. apterus* individual and an experimental time of 24 h were used as standard conditions in the following experiments, if not specified otherwise. The mortality stimulation observed in the EPNs + Pyrap-AKH treatment was further confirmed using firebugs with reduced *Akhr* expression (Fig. 3B). Both *Akhr* knocked down groups showed a reduction in mortality (by 1.6 to 2.9 folds) after EPNs and EPNs + Pyrap-AKH treatments (within 24 and 48 h), in relation to corresponding control samples (with normal *Akhr* expression) (Fig. 3B).

The factors controlling the stimulatory Pyrap-AKH effect on the EPN-induced mortality, how AKH interacts with infection responses in the firebug body, and the mechanism by which AKH increases the mortality induced by EPNs, were also explored. The hypothesis regarding metabolism intensification after AKH injection resulting in a higher turnover of metabolites was tested, considering carbon dioxide production by experimental firebugs as indicative of metabolism intensity. Although the application of EPNs or Pyrap-AKH slightly increased carbon dioxide production, and these were not significantly different from the Ringer-control group (Fig. 4A), the co-application of EPNs + Pyrap-AKH led to a significant increase in carbon dioxide production, which was about 2.1, 1.6 and 1.4 times higher than that in Ringer-control, EPNs treated or Pyrap-AKH treated groups, respectively (Fig. 4A). No carbon dioxide production increase was observed after EPNs + Pyrap-AKH co-application in firebugs with reduced *Akhr* expression (Fig. 4B, C).

3.4. The effect of EPNs on Pyrap-Akh gene expression, and on AKH amount in the CNS and AKH level in haemolymph

The EPN treatments are likely to cause a severe stress in *P. apterus*, activating nervous and endocrine systems and the corresponding biochemical and physiological responses. This theory was evaluated based on *Pyrap-Akh* gene expression in the firebug CNS, and by determining AKH amount in the CNS and AKH level in haemolymph, using competitive ELISA tests (Figs. 5 and 6). A significant increase in *Pyrap-Akh* gene expression (1.6-fold) and a slight but significant increase (1.3-fold) of the AKH amount were detected in the CNS, 24 h after infection (Fig. 5). The response in haemolymph - prepurified by HPLC (Fig. 6A, B) and quantified by ELISA (Fig. 6.C) - was similar, although slightly more intense, and a significant increase in the AKH level (about 1.7-fold) was also observed (Fig. 6C).

3.5. The effect of EPNs on haemolymph nutrient levels

Infection by EPNs and the subsequent release of natural AKHs from the corpora cardiaca into the haemolymph might mobilise available nutrients, primarily from the fat body. Indeed, the EPN treatment elicited a significant increase of total lipid level in the haemolymph (about 1.6-fold, Fig. 7A). A similar but slightly lower

reaction was observed in firebugs with reduced *Akhr* expression (Fig. 7A), and identical trends were obtained for free carbohydrates in the haemolymph after the EPN infection (Fig. 7B). Remarkably, trehalose was significantly mobilized after EPN infection in the control group, but not in *Akhr*-knocked down groups (Fig. 7C).

4. Discussion

The co-application of AKH and several insecticides (permethrin, endosulfan, malathion, pirimiphos-methyl, deltamethrin) has recently been shown to dramatically increase the efficacy of insecticides both in the non-pest insect species *P. apterus* and in the pest *Tribolium castaneum* (Kodrík et al., 2010, 2015b; Velki et al., 2011; Plavšin et al., 2015). Such treatments substantially increased mortality (e.g., from 30% to 91.5% using endosulfan, Velki et al., 2011) and changed several biochemical and physiological characteristics such as total metabolism, total antioxidative capacity, and enzymatic activities, including those of catalase, glutathione-S-transferase, or superoxide dismutase. The most important finding of the present study is that the co-application of *S. carpocapsae* and AKH significantly increased the mortality of firebug individuals and their metabolism. These findings were confirmed in firebugs with reduced *Akhr* expression, treated with EPNs + AKH, in which the mortality was significantly lower and metabolism was not enhanced. Results also suggested that metabolism enhancement elicited by EPN + AKH co-application (compared with application of EPNs alone) in the control (AKHR-normal) group was critical in the process: the higher metabolic turnover might have intensified EPN and bacterial toxin penetration in cells and tissues, and more effectively targeted biochemical and physiological activities in treated insect body. However, increasing metabolism might also lead to faster toxin degradation, but this probably occurs too late, after the toxic effects have been produced; therefore, this mechanism, on its own, is not sufficient to lower mortality. On the other hand, only a negligible effect of AKH alone on the firebug metabolism was recorded. This is not so surprising, because numerous examples exist to show that the effect of AKH in insect body is manifested only in the presence of a stressor: anti-immune response elicited by AKH in *Locusta migratoria* was activated only in presence of immunogen (Goldsworthy et al., 2002a), positive correlation between the hyperlipaemic effect of AKH and its stimulation of locomotor activity was recorded in *P. apterus* only when AKH was applied via injection but not when AKH was applied topically (Kodrík et al., 2002b), and finally the effect of insecticides and AKHs on intensity of insect metabolism mentioned above (Kodrík et al., 2010; Velki et al., 2011; Plavšin et al., 2015) also belong to this category.

During infection, EPNs produce a variety of toxins in the host body, which originate from both nematodes and symbiotic bacteria (Simões et al., 2000; Duchaud et al., 2003). These toxins protect nematodes from the host defence system, but ultimately, they kill the host and transform its tissues into nutrients, which are available for the nematode progeny. Several key regulators and effectors participate in insect responses to nematobacterial infection (Wang et al., 2010; Hyršl et al., 2011; Dobeš et al., 2012; Vojtek et al., 2014; Arefin et al., 2014), especially fast-reacting immune factors and systems, such as those in the clotting cascade. Insects' reactive metabolites, which are produced during oxidative stress and modulated by AKHs (Krishnan and Kodrík, 2012), are other potential factors playing an important role in the interaction between insects and nematobacterial entomopathogens. However, the details of this interaction are still unknown.

EPNs and their symbionts employ various strategies to actively destroy or manipulate insect cellular and humoral immunodefence mechanisms, at the innate immune response or early-induced response stages (Gotz et al., 1981). This involves the secretion of several enzymes, including proteases (Li et al., 2007), phenoloxidase inhibitors, and toxins that interfere with phagocytosis (Sicard et al., 2008; Hao et al., 2008). Secreted enzymes facilitate parasite penetration into the host haemocoel and counteract the insect defence system (Kaya and Gaugler, 1993), and are produced by symbiotic bacteria (Forst et al., 1997) and/or nematodes (Jing et al., 2010). Proteases, for example, are able to destroy the antibacterial peptides expressed by insects in response to EPN infection (Gotz et al., 1981).

In the present study, *P. apterus* infection by *S. carpocapsae* significantly increased the levels of lipids, free carbohydrates, and trehalose in the insect haemolymph (Fig. 7). The increase of these basic energy molecules might have resulted from nematode or bacterial activities (e.g., from the release of digestive enzymes into the host body), or might be a secondary reaction of the firebug body to increasing AKH levels, or both. Mobilization of lipids and free carbohydrates was also observed in firebugs with reduced *Akhr* expression, suggesting the direct activities of the pathogens. However, the total increase in the latter firebug groups was slightly lower than that in the controls, indicating AKHs have a role in the mobilization of lipids and free carbohydrates. Furthermore, *P. apterus* is known to rely on lipids for energy (Kodrík et al., 2000) and its mobilization of carbohydrates after AKH injection is negligible (Socha et al., 2004). However, the extraordinary mobilization of stored energy under the severe stress caused by EPN infection cannot be excluded, and this might explain the fluctuations in trehalose levels. Nevertheless, the impact of this mobilization on total nutrient level is apparently small because of relatively low level of carbohydrates in the firebug haemolymph (Socha et al., 2005; this study: see y-axis scales in Fig. 7A–C). The increased level of nutrients (lipids) found during EPN infection might also explain the increase of EPN-elicited mortality after the AKH treatment, as the AKH-mobilized nutrients might enrich the haemolymph and provide high-quality substrate for the propagation of EPNs and their bacteria. Similar reactions have been described by Goldsworthy et al., 2005, and by Mullen and Goldsworthy (2006), who found that injected AKH increased the mortality of the locust *L. migratoria*, infected with the entomopathogenic fungus *Metarhizium anisopliae* or with the living gram-positive bacterium *Bacillus megaterium*.

Amounts of AKHs, which are usually measured in insect corpora cardiaca, where the stress effect is rather variable, fluctuate under stress conditions, and are probably species-specific and dependant on the type of stressor. For example, application of hydrogen peroxide increased the AKH amount in the firebug CNS about 2.8 times (Bednářová et al., 2013c), while applying paraquat (an herbicide deriving from 4,4′bipyridyl, which induces oxidative stress) elicited no effect (Večeřa et al., 2007). Nevertheless, the latter compound doubled the AKH amount in the fruit fly *D. melanogaster* CNS, although not affecting *Akh* gene expression (Zemanová et al., 2016). Interestingly, a significant increase in *Pyrap-Akh* gene expression after the nematode treatment was recorded in the present study. In addition, the application of insecticides (Kodrík et al., 2015b) and other toxins (Kodrík et al., 2007) usually stimulated AKH synthesis in insect CNS, although producing variable amounts of the hormone. Furthermore, the above-mentioned stressors always significantly up-regulated AKH in insect haemolymph. These results support the assumption that AKH biosynthesis and release coupling is weak or null (Diederen et al., 2002), which is probably related to the large differences in AKH amounts between the corpora cardiaca and the haemolymph: in *P. apterus*, for example, these amounts differ about 200-folds (Kodrík et al., 2003). Thus, AKH requirements in insect haemolymph are easily fulfilled using AKH stocks without immediately affecting AKH synthesis. In the present study, infecting firebugs with *S. carpocapsae* increased

the CNS AKH amount about 1.3 times and haemolymph AKH level about 1.7 times, nevertheless, the absolute increase was much higher in the CNS/corpora cardiaca (in pmols) than in haemolymph (in fmols), regarding the above-mentioned relationships between AKH level in CNS and haemolymph (Diederen et al., 2002; Kodrík et al., 2003).

The important control exerted by AKHs in the general immunity of insects was first described in *L. migratoria* (Goldsworthy et al., 2002a), where injecting laminarin (β-1,3-glucan from fungal cell walls) activated the prophenoloxidase cascade in the haemolymph; this activation was even more intense when AKH was co-applied with laminarin. Injecting a lipopolysaccharide from *Escherichia coli* did not stimulate phenoloxidase activity in the haemolymph, but elicited the formation of nodules; however, the co-injection of the lipopolysaccharide and AKH resulted in the activation of the prophenoloxidase cascade and in the formation of a higher number of nodules (Goldsworthy et al., 2003a,b). Still, it is not known if the same responses occur when AKH is co-applied with EPNs.

In summary, the present study demonstrated that the co-application of *S. carpocapsae* and AKH significantly increased mortality in the firebugs *P. apterus*, compared to the application of nematodes alone. Although the mechanism underlying AKH action is not known, results suggested that the increase in metabolism and nutrient mobilization exerted by this hormone might play a role. In addition, nematobacterial infection increased the amount of AKH in the firebug CNS and haemolymph. Although we are far from detailed understanding of AKH physiological or biochemical mechanisms under stress situations at present, their intensive study might bring interesting results potentially usable also in pest management strategies.

Acknowledgements

This study was supported by grant No. 17-03253S (DK) from the Czech Science Foundation, and by projects RVO 60077344 of the Institute of Entomology. The stay of EI in the University of South Bohemia was supported by the Missions Department in Cairo, Egypt. The authors thank Dr. Z. Mráček for providing of the nematodes *S. carpocapsae*, Miss/Mrs D. Hlávková, H. Štěrbová and L. Kr opáčková for their technical assistance, and Mr. M. Pivarči for the phylogenetic analysis. The English grammar and stylistics were checked by the Editage Author Services.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jinsphys.2017.02.009.

References

Arefin, B., Kučerová, L., Dobeš, P., Markus, R., Strnad, H., Wang, Z., Hyršl, P., Zurovec, M., Theopold, U., 2014. Genome-wide transcriptional analysis of *Drosophila* larvae infected by entomopathogenic nematodes shows involvement of complement, recognition and extracellular matrix proteins. J. Inn. Immun. 6, 192–204.

Bajgar, A., Jindra, M., Doležel, D., 2013. Autonomous regulation of the insect gut by circadian genes acting downstream of juvenile hormone signaling. Proc. Natl. Acad. Sci. U.S.A. 110, 4416–4421.

Beckage, N.E., 1985. Endocrine interactions between endo-parasitic insects and their hosts. Ann. Rev. Entomol. 30, 371–413.

Beckage, N.E., Gelman, D.B., 2001. Parasitism of *Manduca sexta* by *Cotesia congregate*: a multitude of disruptive endocrine effect. In: Edwards, J.P., Weaver, R.J. (Eds.), Endocrine Interactions of Insect Parasites and Pathogens. BIOS Scientific Publishers Ltd, Oxford, England, pp. 59–81.

Bednářová, A., Kodrík, D., Krishnan, N., 2013a. Adipokinetic hormone exerts its anti-oxidative effects using a conserved signal- transduction mechanism involving both PKC and cAMP by mobilizing extra- and intracellular Ca^{2+} stores. Comp. Biochem. Physiol. C 158, 142–149.

Bednářová, A., Kodrík, D., Krishnan, N., 2013b. Unique roles of glucagon and glucagon-like peptides: Parallels in understanding the functions of adipokinetic hormones in stress responses in insects. Comp. Biochem. Physiol. 164, 91–100.

Bednářová, A., Krishnan, N., Cheng, I.C., Večeřa, J., Lee, H.J., Kodrík, D., 2013c. Adipokinetic hormone counteracts oxidative stress elicited in insects by hydrogen peroxide: in vivo and in vitro study. Physiol. Entomol. 38, 54–62.

Bil, M., Broeckx, V., Landuyt, B., Huybrechts, R., 2014. Differential peptidomics highlights adipokinetic hormone as key player in regulating digestion in anautogenous flesh fly, *Sarcophaga crassipalpis*. Gen. Comp. Endocrinol. 208, 49–56.

Bodláková, K., Jedlička, P., Kodrík, D., 2017. Adipokinetic hormones control amylase activity in the cockroach (Periplaneta americana) gut. Insect Sci. http://dx.doi.org/10.1111/1744-7917.12314.

Borovsky, D., Nauen, R., 2007. Biological and biochemical effects of organo-synthetic analogues of trypsin modulating oostatic factor (TMOF) on *Aedes aegypti*, *Heliothis virescens* and *Plutella xylostella*. Pestycydy 3–4, 17–26.

Carroll, N.V., Longley, R.W., Roe, J.H., 1956. The determination of glycogen in liver and muscle by use of anthrone reagent. J. Biol. Chem. 220, 583–593.

Castillo, J.C., Reynolds, S.E., Eleftherianos, I., 2011. Insect immune responses to nematode parasites. Trends Parasitol. 27, 537–547.

Ciche, T.A., Ensign, J.C., 2003. For the insect pathogen *Photorhabdus luminescens*, which end of a nematode is out? Appl. Environ. Microbiol. 69, 1890–1897.

Diederen, J.H.B., Oudejans, R.C.H.M., Harthoorn, L.F., Van der Horst, D.J., 2002. Cell biology of the adipokinetic hormone-producing neurosecretory cells in the locust corpus cardiacum. Microsc. Res. Tech. 56, 227–236.

Dobeš, P., Wang, Z., Markus, R., Theopold, U., Hyršl, P., 2012. An improved method for nematode infection assays in *Drosophila* larvae. Fly 6, 75–79.

Doležel, D., Šauman, I., Košťál, V., Hodková, M., 2007. Photoperiodic and food signals control expression pattern of the clock gene, period, in the linden bug, *Pyrrhocoris apterus*. J. Biol. Rhythms 22, 335–342.

Duchaud, E. et al., 2003. The genome sequence of the entomopathogenic bacterium Photorhabdus luminescens. Nat. Biotechnol. 21, 1307–1313 (25 co-authors).

Forst, S., Dowds, B., Boemare, N., Stackebrandt, E., 1997. *Xenorhabdus* and *Photorhabdus* spp.: Bugs that kill bugs. Annu. Rev. Microbiol. 51, 47–72.

Gäde, G., Auerswald, L., 2003. Mode of action of neuropeptides from the adipokinetic hormone family. Gen. Comp. Endocrinol. 132, 10–20.

Gäde, G., Goldsworthy, G.J., 2003. Insect peptide hormones: a selective review of their physiology and potential application for pest control. Pest Manag. Sci. 59, 1063–1075.

Gäde, G., Hoffmann, K.H., Spring, J.H., 1997. Hormonal regulation in insects: facts, gaps, and future directions. Physiol. Rev. 77, 963–1032.

Goldsworthy, G.J., Chandrakant, S., Opoku-Ware, K., 2003a. Adipokinetic hormone enhances nodule formation and phenoloxidase activation in adult locusts injected with bacterial lipopolysaccharide. J. Insect Physiol. 49, 795–803.

Goldsworthy, G.J., Opoku-Ware, K., Mullen, L.M., 2002a. Adipokinetic hormone enhances laminarin and bacterial lipopolysaccharide-induced activation of the prophenoloxidase cascade in the African migratory locust, *Locusta migratoria*. J. Insect Physiol. 48, 601–608.

Goldsworthy, G.J., Opoku-Ware, K., Mullen, L.M., 2005. Adipokinetic hormone and the immune responses of locusts to infection. Ann. New York Acad. Sci. 1040, 106–113.

Goldsworthy, G.J., Kodrík, D., Comley, R., Lightfoot, M., 2002b. A quantitative study of the adipokinetic hormone of the firebug, *Pyrrhocoris apterus*. J. Insect Physiol. 48, 1103–1108.

Goldsworthy, G.J., Mullen, L.M., Opoku-Ware, K., Chandrakant, S., 2003b. Interactions between the endocrine end immune systems in locusts. Physiol. Entomol. 28, 54–61.

Gotz, P., Boman, A., Boman, H.G., 1981. Interaction between insect immunity and an insect-pathogenic nematode with symbiotic bacteria. Proc. R. Soc. Lond. 212, 333–350.

Grewal, P.S., Ehlers, R.U., Shapiro-Ilan, D.I., 2005. Nematodes as Biological Control Agents. CABI Publishing, Wallingford, UK, p. 528.

Hao, Y.J., Montiel, R., Nascimento, G., Toubarro, D., Simoes, N., 2008. Identification, characterization of functional candidate genes for host-parasite interactions in entomopathogenetic nematode *Steinernema carpocapsae* by suppressive subtractive hybridization. Parasitol. Res. 103, 671–683.

Herbert, E.E., Goodrich-Blair, H., 2007. Friend and foe: the two faces of *Xenorhabdus nematophila*. Nat. Rev. Microbiol. 5, 634–646.

Hyršl, P., Dobeš, P., Wang, Z., Hauling, T., Wilhelmsson, C., Theopold, U., 2011. Clotting factors and eicosanoids protect against nematode infections. J. Innate Immun. 2, 65–70.

Jing, Y.J., Toubarro, D., Hao, Y.J., Simões, N., 2010. Cloning, characterisation and heterologous expression of an astacin metalloprotease, Sc-AST, from the entomoparasitic nematode *Steinernema carpocapsae*. Mol. Biochem. Parasitol. 174, 101–108.

Kaya, H.K., Gaugler, R., 1993. Entomopathogenic nematodes. Ann. Rev. Entomol. 38, 181–206.

Kodrík, D., 2008. Adipokinetic hormone functions that are not associated with insect flight. Physiol. Entomol. 33, 171–180.

Kodrík, D., Bártů, I., Socha, R., 2010. Adipokinetic hormone (Pyrap-AKH) enhances the effect of a pyrethroid insecticide against the firebug *Pyrrhocoris apterus*. Pest Manage. Sci. 66, 425–431.

Kodrík, D., Krishnan, N., Habuštová, O., 2007. Is the titer of adipokinetic peptides in *Leptinotarsa decemlineata* fed on genetically modified potatoes increased by oxidative stress? Peptides 28, 974–980.

Kodrík, D., Socha, R., Syrová, Z., 2003. Developmental and diel changes of adipokinetic hormone in CNS and haemolymph of the flightless wing-polymorphic bug, *Pyrrhocoris apterus*. J. Insect Physiol. 49, 53–56.

Kodrík, D., Socha, R., Zemek, R., 2002a. Topical application of Pya-AKH stimulates lipid mobilization and locomotion in the flightless bug, *Pyrrhocoris apterus* (L.) (Heteroptera). Physiol. Entomol. 27, 15–20.

Kodrík, D., Bednářová, A., Zemanová, M., Krishnan, N., 2015a. Hormonal regulation of response to oxidative stress in insects - an update. Int. J. Mol. Sci. 16, 25788–25816.

Kodrík, D., Plavšin, I., Velki, M., Stašková, T., 2015b. Enhancement of insecticide efficacy by adipokinetic hormones. In: Montgomery, J. (Ed.), Insecticides: Occurrence, Global Threats and Ecological Impact. Nova Science Publishers Inc, New York, pp. 77–91.

Kodrík, D., Šimek, P., Lepša, L., Socha, R., 2002b. Identification of the cockroach neuropeptide Pea-CAH-II as a second adipokinetic hormone in the firebug *Pyrrhocoris apterus*. Peptides 23, 585–587.

Kodrík, D., Vinokurov, K., Tomčala, A., Socha, R., 2012. The effect of adipokinetic hormone on midgut characteristics in *Pyrrhocoris apterus* L. (Heteroptera). J. Insect Physiol. 58, 194–204.

Kodrík, D., Socha, R., Šimek, P., Zemek, R., Goldsworthy, G.J., 2000. A new member of the AKH/RPCH family that stimulates locomotory activity in the firebug, *Pyrrhocoris apterus* (Heteroptera). Insect Biochem. Mol. Biol. 30, 489–498.

Kodrík, D., Stašková, T., Jedličková, V., Weyda, F., Závodská, R., Pflegerová, J., 2015c. Molecular characterization, tissue distribution, and ultrastructural localization of adipokinetic hormones in the CNS of the firebug *Pyrrhocoris apterus* (Heteroptera, Insecta). Gen. Comp. Endocrinol. 210, 1–11.

Koppenhöfer, A.M., 2007. Nematodes. In: Lacey, L.A., Kaya, H.K. (Eds.), Field Manual of Techniques in Invertebrate Pathology: Application and Evaluation of Pathogens for Control of Insects and Other Invertebrate Pests. Springer, Dordrecht, pp. 249–264.

Krishnan, N., Kodrík, D., 2012. Endocrine control of oxidative stress in insects. In: Farooqui, T., Farooqui, A.A. (Eds.), Oxidative Stress in Vertebrates and Invertebrates: Molecular Aspects of Cell Signaling. Wiley-Blackwell, New Jersey, pp. 261–270.

Lee, G., Park, J.H., 2004. Haemolymph sugar homeostasis and starvation-induced hyperactivity affected by genetic manipulations of the adipokinetic hormone-encoding gene in *Drosophila melanogaster*. Genetics 167, 311–323.

Li, X.Y., Cowles, R.S., Cowles, E.A., Gaugler, R., Cox-Foster, D.L., 2007. Relationship between the successful infection by entomopathogenic nematodes and the host immune response. Int. J. Parasitol. 37, 365–374.

Lorenz, M.W., Zemek, R., Kodrík, D., Socha, R., 2004. Lipid mobilisation and locomotor stimulation in *Gryllus bimaculatus* (de Geer) (Ensifera, Gryllidae) by topically applied adipokinetic hormone. Physiol. Entomol. 29, 146–151.

Mullen, L.M., Goldsworthy, G.J., 2006. Immune responses of locusts to challenge with the pathogenic fungus *Metarhizium* or high doses of laminarin. J. Insect Physiol. 52, 389–398.

Ons, S., Lavore, A., Sterkel, M., Wulff, J.P., Sierra, I., Martinez-Barnetche, J., Rodriguez, M.H., Rivera-Pomar, R., 2016. Identification of G protein coupled receptors for opsines and neurohormones in *Rhodnius prolixus*. Genomic and transcriptomic analysis. Insect Biochem. Mol. Biol. 69, 34–50.

Park, Y., Kim, Y.J., Adams, M.E., 2002. Identification of G protein-coupled receptors for *Drosophila* PRXamide peptides, CCAP, corazonin, and AKH supports a theory of ligand-receptor coevolution. Proc. Natl. Acad. Sci. U.S.A. 99, 11423–11428.

Plavšin, I., Stašková, T., Šerý, M., Smýkal, V., Hackenberger, H.K., Kodrík, D., 2015. Hormonal enhancement of insecticide efficacy in *Tribolium castaneum*: Oxidative stress and metabolic aspects. Com. Biochem. Physiol. C170, 19–27.

Scarborough, R.M., Jamieson, G.C., Kalisz Kramer, S.J., McEnroe, G.A., Miller, C.A., Schooled, D.A., 1984. Isolation and primary structure of two peptides with cardioacceleratory and hyperglycaemic activity from the corpora cardiaca of Periplaneta americana. Proc. Nat. Acad. Sci. U.S.A. 81, 5575–5579.

Shapiro-Ilan, D.I., Dawn, H.G., Simon, J.P., Jane, P.F., 2006. Application technology and environmental considerations for use of entomopathogenic nematodes in biological control. Biol. Control 38, 124–133.

Sicard, M., Raimond, M., Prats, O., Lafitte, A., Braquart-Varnier, C., 2008. Pathogenic effect of entomopathogenic nematode–bacterium complexes on terrestrial isopods. J. Invertebr. Pathol. 99, 20–27.

Simões, N., Caldas, C., Rosa, J.S., Bonifassi, E., Laumond, C., 2000. Pathogenicity caused by high virulent and low virulent strains of *Steinernema carpocapsae* to *Galleria mellonella*. J. Invertebr. Pathol. 75, 47–54.

Socha, R., Kodrík, D., Šula, J., 2005. Wing morph specific differences in the metabolism and endocrine control of reserve mobilization in adult males of a flightless bug, *Pyrrhocoris apterus* (L.) (Heteroptera). J. Comp. Physiol. B 175, 557–565.

Socha, R., Kodrík, D., Šimek, P., Patočková, M., 2004. The kind of AKH-mobilized energy substrates in insects can be predicted without a knowledge of the hormone structure. Eur. J. Entomol. 101, 29–35.

Staubli, F., Jorgensen, T.J.D., Cazzamali, G., Williamson, M., Lenz, C., Sondergaard, L., Roepstorff, P., Grimmelikhuijzem, C.J.P., 2002. Molecular identification of the insect adipokinetic hormone receptors. Proc. Natl. Acad. Sci. U.S.A. 99, 3446–3451.

Urbanová, V., Bazalová, O., Vaněčková, H., Doležel, D., 2016. Photoperiod regulates growth of male accessory glands through juvenile hormone signaling in the linden bug, *Pyrrhocoris apterus*. Insect Biochem. Mol. Biol. 70, 184–190.

Tanaka, Y., Suetsugu, Y., Yamamoto, K., Noda, H., Shinoda, T., 2014. Transcriptome analysis of neuropeptides and G-protein coupled receptors (GPCRs) for neuropeptides in the brown planthopper *Nilaparvata lugens*. Peptides 53, 125–133.

Uvell, H., Engstrom, Y., 2007. A multilayered defense against infection: combinatorial control of insect immune genes. Trends Genet. 23, 342–349.

Večeřa, J., Krishnan, N., Alquicer, G., Kodrík, D., Socha, R., 2007. Adipokinetic hormone-induced enhancement of antioxidant capacity of *Pyrrhocoris apterus* hemolymph in response to oxidative stress. Comp. Biochem. Phys. C146, 336–342.

Veenstra, J.A., Rombauts, S., Grboc, M., 2012. In silico cloning of genes encoding neuropeptides, neurohormones and their putative G-protein coupled receptors in a spider mite. Insect Biochem. Mol. Biol. 42, 277–295.

Velki, M., Kodrík, D., Večeřa, J., Hackenberger, B.K., Socha, R., 2011. Oxidative stress elicited by insecticides: a role for the adipokinetic hormone. Gen. Comp. Endocrinol. 172, 77–84.

Verlinden, H., Vleugels, R., Zels, S., Dillen, S., Lenaerts, C., Crabbe, K., Spit, J., Van den Broeck, J., 2014. Receptors for neuronal or endocrine signalling molecules as potential targets for the control of insect pests. In: Cohen, E.A. (Ed.), Advances in Insect Physiology. Elsevier Science Publishers, London, pp. 167–303.

Vojtek, L., Dobeš, P., Büyükgüzel, E., Atosuo, J., Hyršl, P., 2014. Bioluminescent assay for evaluating antimicrobial activity in insect haemolymph. Eur. J. Entomol. 111, 335–340.

Wang, Z., Wilhelmsson, C., Hyršl, P., Loof, T.G., Dobeš, P., Klupp, M., Loseva, O., Mörgelin, M., Iklé, J., Cripps, R.M., Herwald, H., Theopold, U., 2010. Pathogen entrapment by transglutaminase-A conserved early innate immune mechanism. PLoS Pathog. 6, 1–9.

Waterfield, N.R., Ciche, T., Clarke, D., 2009. *Photorhabdus* and a host of hosts. Annu. Rev. Microbiol. 63, 557–574.

Wicher, D., Agricola, H.J., Sohler, S., Gundel, M., Heinemann, S.H., Wollweber, L., Stengl, M., Derst, C., 2006. Differential receptor activation by cockroach adipokinetic hormones produces differential effects on ion currents, neuronal activity, and locomotion. J. Neurophysiol. 95, 2314–2325.

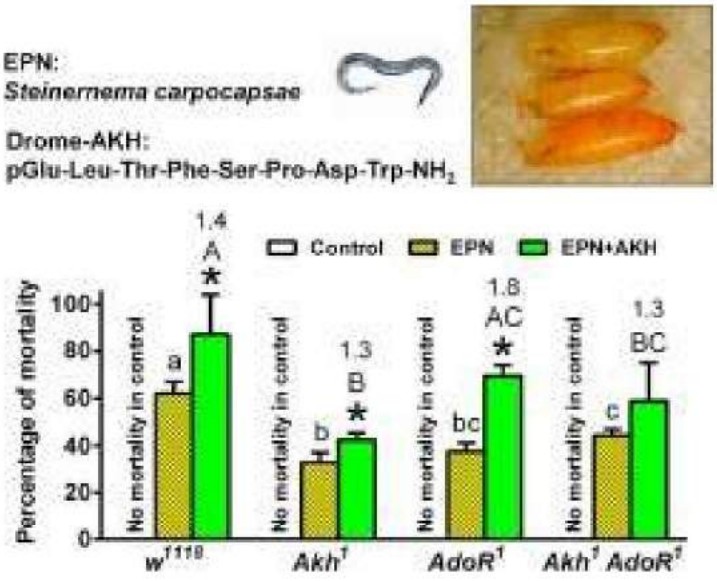
Withers, P.C., 1977. Measurement of V_{O2}, V_{CO2} and evaporative water loss with a flow-through mask. J. Appl. Physiol. 42, 120–123.

Zemanová, M., Stašková, T., Kodrík, D., 2016. Role of adipokinetic hormone and adenosine in the anti-stress response in *Drosophila melanogaster*. J. Insect Physiol. 91–92, 39–47.

Zöllner, N., Kirsch, K., 1962. Über die quantitative Bestimmung von Lipoide (Mikromethode) mittels der vielen natürlichen Lipoiden (allen bekannten Plasmalipoiden) gemeinsamen Sulfo-phospho-vanillin-Reaktion. Z Ges. Exp. Medizin. 135, 545–561.

5.2. Paper 2

Ibrahim E., Dobeš P., Kunc M., Hyršl P., Kodrík D. (2018) Adipokinetic hormone and adenosine interfere with nematobacterial infection and locomotion in *Drosophila melanogaster*. *J. Insect Physiol.* 107: 167-174.



Journal of Insect Physiology 107 (2018) 167–174

Contents lists available at ScienceDirect

Journal of Insect Physiology

journal homepage: www.elsevier.com/locate/jinsphys

Adipokinetic hormone and adenosine interfere with nematobacterial infection and locomotion in *Drosophila melanogaster*

Emad Ibrahim[a,c], Pavel Dobeš[b], Martin Kunc[b], Pavel Hyršl[b], Dalibor Kodrík[a,*]

[a] Institute of Entomology, Biology Centre, CAS, and Faculty of Science, University of South Bohemia, Branišovská 31, 370 05 České Budějovice, Czech Republic
[b] Institute of Experimental Biology, Faculty of Science, Masaryk University, Kotlářská 2, 611 37 Brno, Czech Republic
[c] Faculty of Agriculture, University of Cairo, Giza, Egypt

ARTICLE INFO

Keywords:
Adipokinetic hormone
Adenosine
Drosophila
Nematode
Oxidative stress
Locomotion

ABSTRACT

This study examined how adipokinetic hormone (AKH) and adenosine affect defense responses in *Drosophila melanogaster* larvae infected with entomopathogenic nematodes (EPN, *Steinernema carpocapsae* and *Heterorhabditis bacteriophora*). Three loss-of-function mutant larvae were tested: Akh^1, $AdoR^1$ (adenosine receptor), and Akh^1 $AdoR^1$. Mortality decreased in all mutants post-EPN infection compared with the control (w^{1118}). Additionally, co-application of external AKH with EPN significantly increased mortality beyond rates observed in EPN-only treatment, while also elevating carbon dioxide production, a measure of metabolism. Furthermore trehalose levels increased in both w^{1118} and Akh^1 larvae post-EPN infection, but the latter group exhibited a lower increase and total trehalose levels. Interestingly, baseline trehalose was relatively high in untreated $AdoR^1$ and Akh^1 $AdoR^1$ mutants, with levels remaining unaffected by infection. Infection also elevated haemolymph lipid content overall, but the different mutations did not substantially influence this change. In contrast, haemolymph protein content dropped after EPN infection in all tested groups, but this decline was more intense among Akh^1. In uninfected larvae mutations decreased antioxidative capacity in Akh^1 and increased in $AdoR^1$, however, its post-infection increases were similar in all mutants, suggesting that antioxidant response in *Drosophila* involves mechanisms also beyond AKH and adenosine. Furthermore, AKH application in w^{1118} larvae significantly increased movement distance and percentage of larval activity, but reduced velocity. Mutations of *Akh* and *AdoR* did not strongly affect locomotion.

1. Introduction

In insects, unfavourable stressors that disrupt physiological homeostasis activate defense responses, predominantly under the control of adipokinetic hormones (AKHs). Part of the AKH/RPCH neuropeptide family (Gäde et al., 1997), AKH is approximately 8–10 amino acids in size and its signal transduction pathway is well documented in the fat body (Gäde and Auerswald, 2003). Synthesis, storage, and secretion of AKHs occur either in neurosecretory cells from the corpora cardiaca, a neuroendocrine gland connected to the brain, or in corresponding corpora cardiaca cells of dipteran ring gland. In some insect species, AKHs have been found in the brain itself, suggesting a role in neuronal signalling there (Milde et al., 1995; Kodrík et al., 2003, 2015a), although unequivocal evidence remains lacking.

Although AKH function is pleiotropic, they are generally involved in energy metabolism (Kodrík, 2008), specifically in catabolic reactions that mobilize lipids, carbohydrates, and proline (Gäde et al., 1997). In *Drosophila melanogaster*, ablation of AKH-secreting cells (Isabel et al., 2005) or *Akh* mutations (Sajwan et al., 2015) significantly decrease circulating carbohydrate levels. Likewise, in the firebug *Pyrrhocoris apterus*, AKH injection significantly increases haemolymph lipid levels (Kodrík et al., 2000). These hormones also stimulate heartbeat (Scarborough et al., 1984), general locomotion (Kodrík et al., 2000), food intake (Kodrík et al., 2012), gut digestion (Bil et al., 2014; Bodláková et al., 2017, 2018) and feeding behaviour (Lee and Park, 2004). Under certain conditions, AKH interaction with humoral and cellular immune systems involves activating the prophenoloxidase cascade and nodule formation (Goldsworthy et al., 2002, 2003). Moreover, AKHs are involved in defense against oxidative stress (Kodrík et al., 2007). Experiments have shown that AKHs restore antioxidative stress reactions in insect tissues and *in vitro* organ cultures that were experimentally stressed (reviewed by Krishnan and Kodrík, 2012; Kodrík et al., 2015b).

As mentioned above AKH plays a significant role in control of insect skeletal and visceral muscles, and in general locomotion (Kodrík, 2008; Lorenz and Gäde, 2009). The first pioneer studies revealed that AKH

* Corresponding author.
E-mail address: kodrik@entu.cas.cz (D. Kodrík).

https://doi.org/10.1016/j.jinsphys.2018.04.002
Received 9 March 2018; Received in revised form 4 April 2018; Accepted 4 April 2018
Available online 05 April 2018

injection stimulates frequency of spontaneous contractions of isolated leg muscles in a locust, and frequency of heartbeat in the cockroach *Periplaneta americana* (O'Shea et al., 1984; Scarborough et al., 1984; Witten et al., 1984). Later on, the stimulatory effect of AKH on insect locomotor activity has been documented in several insect species as *P. apterus* (Socha et al., 1999; Kodrík et al., 2000), *Gryllus bimaculatus* (Lorenz et al., 2004) or *P. americana* (Wicher et al., 2006). In *Drosophila* ablation of AKH producing cells by specifically expressed apoptosis transgene, resulted in hypoactive adults (Isabel et al., 2005). Similarly, flies without AKH-producing neurons, did not exhibit the increase of locomotor activity recorded in starved control flies prior to their death (Lee and Park, 2004); this suggests an AKH positive role in induction of a foraging behaviour under the shortage of energy reserves.

Also important to insect stress response is the purine nucleoside adenosine, a paracrine and systemic signalling molecule of metabolic imbalance. Adenosine accumulates extracellularly in response to cell damage, hypoxia, parasitization, or metabolic stress (Csóka and Haskó, 2011). Like AKH, adenosine is involved in regulating energy metabolism, antioxidation, immune response, adipose tissue formation, and neural functions (Jacobson and Gao, 2009; Kodrík, 2008; Zemanová et al., 2016). Unsurprisingly given their overlap in function, adenosine and AKH signalling pathways also overlap in composition. For example, G-protein coupled receptors (GPCRs) mediate both pathways through modulating cAMP production (Park et al., 2002; Jacobson and Gao, 2009).

Entomopathogenic nematodes (EPN) are parasites that form highly pathogenic complexes with mutualistic bacteria. Upon infection, EPNs release their associated bacteria into the insect body cavity, and both components of the complex produce multiple toxins (Simões et al., 2000; Duchaud et al., 2003; Lu et al., 2017). These EPNs are thus a useful stressor for examining insect defense response. Previously, we identified several key regulators and effectors of response to nematobacterial infection (Wang et al., 2010; Hyršl et al., 2011; Arefin et al., 2014; Jančaříková et al., 2017), but little is known about how these factors interact with the insect endocrine system. Intriguingly, some hints are available from data on insecticide efficacy. Topical application (Kodrík et al., 2002) or injection of AKHs enhances insecticidal effects (Kodrík et al., 2010; Velki et al., 2011; Plavšin et al., 2015; reviewed by Kodrík et al., 2015c), possibly because AKHs accelerate metabolite exchange that then causes faster insecticide penetration into tissues. A lead-up study to this current work demonstrated in the firebug, *P. apterus* that EPN infection increases AKH and modulates some biochemical characteristics, such as a level of lipids, trehalose or free carbohydrates (Ibrahim et al., 2017). Thus, if AKHs are overproduced in response to highly stressful EPN attack, insects may actually experience greater mortality.

The fruit fly model *D. melanogaster* is ideal for research on insect defence mechanisms under EPN attack; the *Drosophila* AKH amino-acid sequence is available (pGlu-Leu-Thr-Phe-Ser-Pro-Asp-Trp-NH_2; Schaffer et al., 1990) and suitable loss-of-function mutants are readily prepared. Here, we used the fruit fly to verify our earlier findings (Ibrahim et al., 2017), as well as expand our existing understanding of AKH and adenosine function in defense against EPN. Furthermore, we studied how AKH and adenosine affect *Drosophila* locomotion and whether there is any overlap between these two signalling systems which remains unknown so far.

2. Material and methods

2.1 Experimental flies

The fruit flies *D. melanogaster* were maintained in glass vials with standard corn meal/yeast/sucrose/agar diet at 25 °C and 12:12 L:D cycles. All assays were carried out on larvae of the 3rd instar, 3-day after the larval hatching at same temperature and light conditions. The fly strains used in this study were as followings: (1) the controls w^{1118} (Bloomington Centre); (2) Akh^1 mutant with a three bases deletion in the *Akh* gene on the chromosome III resulting in lack of the third amino acid in AKH octapeptide (Sajwan et al., 2015); (3) $AdoR^1$ mutant carrying the insertion of mini-white, w^{+mC}, in *AdoR* gene (Doležal et al., 2005); and (4) Akh^1 $AdoR^1$ double mutant carrying both the deletion in the *Akh* gene and the w^{+mC} insertion in *AdoR* gene, which was prepared by crossing of homozygous strains of mutants in the adenosine receptor ($AdoR^1$) and adipokinetic hormone (Akh^1) and making a recombinant (Zemanová et al., 2016).

2.2 Entomopathogenic nematodes

Two nematode species *Steinernema carpocapsae* and *Heterorhabditis bacteriophora* with symbiotic bacteria *Xenorhabdus nematophila* and *Photorhabdus luminescens*, respectively, were used in the study.

The nematodes *S. carpocapsae* originating from Russia (strain NCR), St. Petersburg and *H. bacteriophora* (strain H222) collected in Pouzdřany, Czech Republic were kindly provided by 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 of *S. carpocapsae* were harvested and subsequently stored in autoclaved water at 4 °C for 30 days in the dark. *H. bacteriophora* was kept at laboratory temperature and used 30 days after harvesting. Viability of both nematode species was confirmed under a microscope before experiments.

2.3 Nematode and hormone treatments

Application of *D. melanogaster* AKH (Drome-AKH): the hormone (Vidia, Praha, Czech Republic) was applied by dipping of the experimental larvae in Drome-AKH solution in 20% methanol (3 pmol per µl) for a few seconds. The effect was evaluated 24 h after the treatment.

Application of nematodes: *Drosophila* larvae were infected with entomopathogenic nematodes as described previously (Dobeš et al., 2012, Arefin et al., 2014). Briefly, nematodes were diluted by autoclaved water to the concentration 100 and 25 infective juveniles (suitable sub-lethal doses) per 10 µl for *S. carpocapsae* and *H. bacteriophora*, respectively, and 10 µl of this suspension was soaked to the piece of paper placed in wells of microtiter plate (96-well, flat bottom). Individual *Drosophila* larvae were transferred to the wells where they stayed in contact with nematodes, and the plate was covered by Parafilm®. Infection was conducted at 25 °C in the dark. Infected larvae were scored after 24 h based on their specific coloration (yellowish or reddish when infected with *S. carpocapsae* or *H. bacteriophora*, respectively) and not responding to the prodding by forceps. Each experimental group consisted of 48 *Drosophila* larvae and all experiment were done in 3–8 repetitions which means at least 144 larvae per infected group. Survived larvae were used for further evaluation of metabolic changes.

2.4 Metabolic rate measuring

A flow-through respirometry system LI-7000 CO_2/H_2O analyser (LI-COR Biosciences, Lincoln, NE, USA) was used to determine the carbon dioxide production rate by the experimental larvae (Kodrík et al., 2010). The measurement was based on the differences of CO_2 content between the air sample passing through the reference, and the air sample in each chamber containing experimental insects. The reference chamber is used for the acquisition of baseline signal and for neutralization of any possible variations in the system. The air was pushed through the experimental chamber (volume 8 mL) at the flow rate of ~80 mL·min^{-1}. The larvae (five individuals per chamber, 4–8 chambers per group) were examined for a period of 40 min. The data was analysed by data acquisition software (ExpeData Software, Sable System, Las Vegas, Nevada, USA). The carbon dioxide production

(V_{CO2}) was calculated from fractional concentrations of carbon dioxide going into (FI) and coming out of (FE) of the respirometry chamber using an equation according to Withers (1977) and expressed in µL per min^{-1} mg^{-1} units:

$V_{CO2} = (FE_{CO2} - FI_{CO2})$ f, where 'f' is the flow rate in µL h^{-1}.

2.5 AKH extraction and level determination by ELISA

The larval heads containing central nervous system with the ring gland attached were cut off and extracted in 80% methanol; the extract was used for the determination of the AKH level using a competitive ELISA as described in our previous paper (Zemanová et al., 2016). Briefly, the 96-well microtiter plates (high binding Costar, Corning Incorporated, Corning, New York) were pre-coated overnight with IgG (anti Drome-AKH polyclonal antibody; a kind gift of Jan Veenstra, Bordeaux University, France), dilution 1:10,000 in coating buffer. After blocking with 3% BSA solution (bovine serum albumin) in dH_2O, the samples were added to specific wells, followed by the biotinylated probe prepared from Drome-AKH using Biotin Long Arm Maleimide (BLAM, Gln-Leu-Thr-Phe-Ser-Pro-Asp-Trp-Lys(Bio)-Gly-NH_2; Vidia, Praha, Czech Republic), diluted in 1 mM PBS (phosphate-buffered saline, pH = 7.5). After the competition for the binding sites on the IgG bound to the plates a streptavidin conjugated with horseradish peroxidase solution (Vector Laboratories, CA, USA), diluted 1:500 in PBS-Tween was added to each well. Finally, the ELISA substrate (3,3′,5,5′-tetramethylbenzidine, Sigma Aldrich) was added and after the visualization the reaction was stopped by 0.5 M sulphuric acid. The absorbance values were determined in a microtiter plate reader at 450 nm. The known concentration of Drome-AKH was used as a standard which allowed construction of a competition curve and estimation of the AKH content in analysed samples. The detection limit of this ELISA test was estimated from the standard curve to be about 15 fmol Drome-AKH per well (data not shown).

2.6 Spectrophotometric determination of nutrients

The level of trehalose, lipids and proteins was determined in the larval haemolymph 24 h after the *S. carpocapsae* treatment. For this, the larvae were pierced by forceps, and haemolymph was taken from them by gentle centrifugation. The haemolymph was diluted 3 times in potassium phosphate buffer (50 mM, pH 7.0), centrifuged (10,000*g*, 10 min, 4 °C) and 1 µl of supernatant per sample was used for determination of the nutrients.

- *Trehalose determination* – for trehalose quantification, the reducing sugars were removed from the samples, and then trehalose itself was determined using the Trehalose assay kit, (Megazyme) according to the manufacturer's instructions. Briefly, 10 µl samples (1 µl haemolymph supernatant and 9 µl water) were mixed with an equivalent volume of alkaline borohydrate solution (10 mg/ml sodium borohydrate in 50 mM sodium hydroxide) and incubated for 30 min at 40 °C. Then, the excess of borohydride was removed with 25 µl of 200 mM acetic acid; 5 min later, 10 µl of 2 M imidazole buffer (pH 7.0) was added to adjust the pH level in the sample to the neutral. The trehalose level was determined in 27.5 µl samples using the anthrone method (Carrol et al., 1956) that was modified by Socha et al. (2004).
- *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, Japan), were converted to µg lipids per µl haemolymph with the aid of a calibration curve based on known amounts of oleic acid.
- *Protein determination* – was done by Bicinchoninic Acid Protein Assay Kit (Sigma Aldrich) (Stoscheck, 1990). One µl of haemolymph supernatant was added to 400 µl of Ringer saline, and 10 µl sample was mixed with 300 µl of bicinchoninic acid containing $CuSO_4 \cdot 5H_2O$. The bovine serum albumin standard curve was used to convert the optical densities of the samples measured at 562 nm to µg proteins.

2.7 Total anti-oxidative activity

This oxidative stress marker was determined in the whole body extracts. The extracts were prepared by homogenization of the larval bodies in potassium phosphate buffer (50 mM, pH 7.0) in ratio 3 µl buffer per 1 mg fresh body weight. The homogenate was centrifuged for 10 min at 10,000*g* at 4 °C, supernatant diluted 10 times and used immediately for the assay. The Antioxidant Assay Kit (Sigma Aldrich, CS0790) with appropriate manufacturer protocols was employed.

2.8 Locomotor activity

Locomotor characteristics were monitored using the FIMTrack method, which allows simultaneous tracking of *Drosophila* larvae. This method is based on frustrated total internal reflection of infrared light. The infrared light emitted by FIMTrack table travels through acrylic glass until it strikes *Drosophila* larvae and is reflected to camera with infrared filter (Risse et al., 2017).

The activity of fly larvae was observed in round arena surrounded with salt barrier, and with food in the middle according to Kunc et al. (2017). Typically 8 parallel measurements, with 15 larvae per each, were done for each larval group. Images were captured by Basler ace acA2040-90uc camera in a dark room without any additional light source except the built-in infrared light generated by the FIMTrack. Images were captured with a frequency of 2 frames per second for 720 s in the size of 1200 × 1200 pixels and processed in FIMTrack v2 Windows software (downloaded from https://www.uni-muenster.de/PRIA/en/FIM/). All the fly tracks were at first processed automatically and then manually verified. Measured characteristics included (1) the distance crawled by larvae in 720 s, (2) the velocity of larval movement and (3) the percentage of larval activity (go phase).

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 bar graphs represent mean ± SD, the numbers of replicates (n) are depicted in the figure legends. The statistical differences were evaluated by one-way ANOVA with Tukey's post-hoc test (Figs. 1, 3–8) and by Student's *t*-test (Figs. 1, 2, 4 and 8) using the Prism software.

3. Results

3.1 Mortality and metabolism under nematobacterial infection

Infection with the EPN *S. carpocapsae* resulted in significantly lower mortality among the three loss-of-function mutants compared with w^{1118} (Fig. 1). Mortality those mutants was also lower after EPN and AKH co-application, although the difference was not significant among $AdoR^1$ mutants. Further, EPN + AKH significantly increased mortality by 1.3–1.8 fold in larvae compared with EPN application alone, with highest increase among $AdoR^1$. Infection with *H. bacteriophora* yielded nearly identical results (Fig. S1), except that $AdoR^1$ mutants did not experience a significant increase in mortality under EPN + AKH compared with EPN-only treatment or w^{1118}, while Akh^1 $AdoR^1$ double mutants were the most strongly affected by *H. bacteriophora* and AKH co-application.

Overall similarities in the above data led to the use of *S. carpocapsae* for all subsequent experiments. ELISA results revealed that EPN only negligibly increased AKH in w^{1118} larvae but significantly increased AKH by 1.6 fold in $AdoR^1$ (Fig. 2). As expected, the non-functional AKH

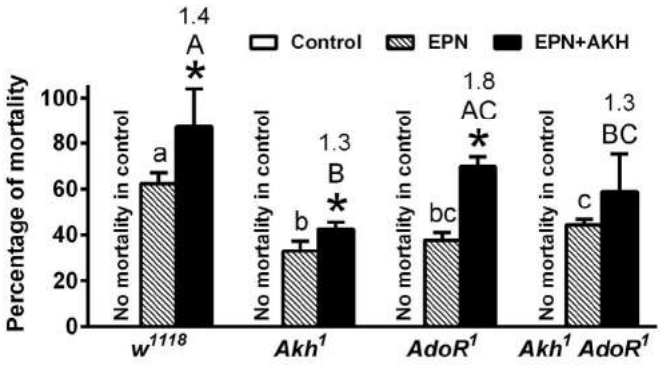


Fig. 1. The effect of entomopathogenic nematode (EPN) *S. carpocapsae* and Drome-AKH (AKH) on mortality of various *D. melanogaster* mutants 24 h after the treatment. Statistically significant differences among the mutants at the 5% level evaluated by one-way ANOVA with Tukey's post-hoc test are indicated by different letters (effect of mutation); differences between the experimental groups (EPN vs. EPN + AKH) within the mutant (effect of treatment) at the 5% level evaluated by Student's *t*-test are indicated by asterisks. The numbers above the columns represent fold-difference of mortality after treatments by EPN with Drome-AKH as compared with the nematode infection only (effect of AKH); n = 4–5, 48 larvae per group.

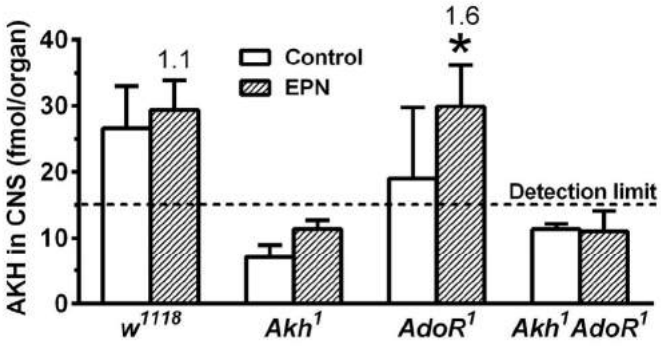


Fig. 2. The effect of entomopathogenic nematode (EPN) *S. carpocapsae* on Drome-AKH level in CNS (including the ring gland) of various *D. melanogaster* mutants 24 h after the treatment. The levels of AKH in the mutants producing deficient AKH – not recognised by used antibody against normal AKH – were under the ELISA detection limit (15 fmol) therefore they were not included into the statistics. Statistically significant differences between the EPN treatment and corresponding untreated controls at the 5% level evaluated by Student's *t*-test are indicated by asterisks (effect of treatment); no significant differences were recorded between the $AdoR^1$ mutant and w^{1118} control (both for treated and untreated couples; again using Student's *t*-test). The numbers above the columns represent fold-difference of AKH level after treatments by *S. carpocapsae* as compared with untreated controls; n = 5–8.

of both Akh^1 mutants was undetectable with the ELISA antibody.

We next examined how EPN-only and EPN + AKH treatments influenced carbon dioxide production as a measure of total metabolism (Fig. 3). (We did not test AKH-only treatments because we have already demonstrated that this effect is not significant (Kodrík et al., 2010). We found that EPN significantly decreased carbon dioxide production in all larvae except $AdoR^1$, whereas EPN + AKH significantly increased production. This latter increase was higher in AKH loss-of-function mutants (Akh^1: 3.4 fold, Akh^1 $AdoR^1$: 4.8 fold), moderate in w^{1118} (2.6 fold) and lowest in $AdoR^1$ larvae (1.5 fold).

We also found that Akh^1 mutants had significantly lower, and $AdoR^1$ and Akh^1 $AdoR^1$ mutants significantly higher trehalose (main carbohydrate in haemolymph) than w^{1118} (Fig. 4), suggesting both AKH and adenosine involvement in modulating trehalose content. After EPN infection, w^{1118} and Akh^1 larvae experienced a 1.9-fold and 1.6-fold significant increase in trehalose, whereas $AdoR^1$ and Akh^1 $AdoR^1$ mutants showed no such increase. Moreover, haemolymph lipids increased

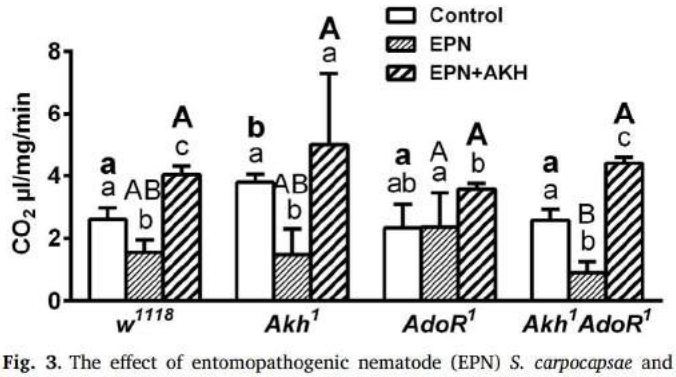


Fig. 3. The effect of entomopathogenic nematode (EPN) *S. carpocapsae* and Drome-AKH (AKH) treatments on carbon dioxide production in the body of various *D. melanogaster* mutants 24 h after the treatment. Statistically significant differences among the mutants at the 5% level evaluated by one-way ANOVA with Tukey's post-hoc test are indicated by different letters according to the following scheme: a – significance within the fly group (i.e. control vs. EPN vs. EPN + AKH), *a – significance within controls*, A – SIGNIFICANCE WITHIN EPN, *A – SIGNIFICANCE WITHIN EPN + AKH*; n = 4–8.

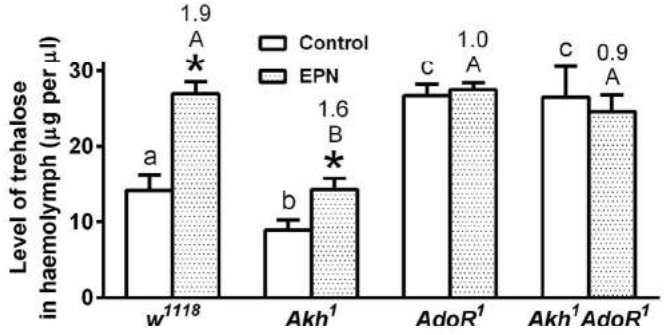


Fig. 4. The effect of entomopathogenic nematode (EPN) *S. carpocapsae* on trehalose level in haemolymph of various *D. melanogaster* mutants 24 h after the treatment. Statistically significant differences among the mutants at the 5% level evaluated by one-way ANOVA with Tukey's post-test are indicated by different letters (effect of mutation); differences between the experimental groups within the mutant (effect of treatment) at the 5% level evaluated by Student's *t*-test are indicated by asterisks. The numbers above the columns represent fold-difference of trehalose level after the treatment by *S. carpocapsae* as compared with the controls; n = 3–8.

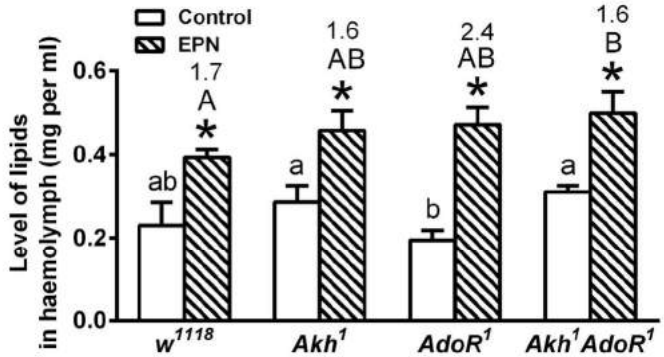


Fig. 5. The effect of entomopathogenic nematode (EPN) *S. carpocapsae* on lipid level in haemolymph of various *D. melanogaster* mutants 24 h after the treatment. Statistically significant differences among the mutants at the 5% level evaluated by one-way ANOVA with Tukey's post-test are indicated by different letters (effect of mutation); differences between the experimental groups within the mutant (effect of treatment) at the 5% level evaluated by Student's *t*-test are indicated by asterisks. The numbers above the columns represent fold-difference of lipid level after treatments by *S. carpocapsae* as compared with the controls; n = 4–5.

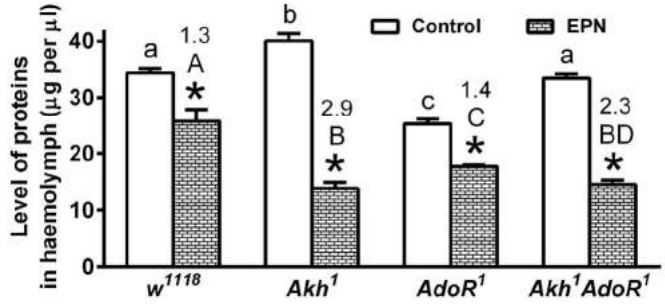


Fig. 6. The effect of entomopathogenic nematode (EPN) *S. carpocapsae* on protein level in haemolymph of various *D. melanogaster* mutants 24 h after the treatment. Statistically significant differences among the mutants at the 5% level evaluated by one-way ANOVA with Tukey's post-test are indicated by different letters (effect of mutation); differences between the experimental groups within the mutant (effect of treatment) at the 5% level evaluated by Student's *t*-test are indicated by asterisks. The numbers above the columns represent fold-difference of protein level after treatments by *S. carpocapsae* as compared with the controls; n = 5.

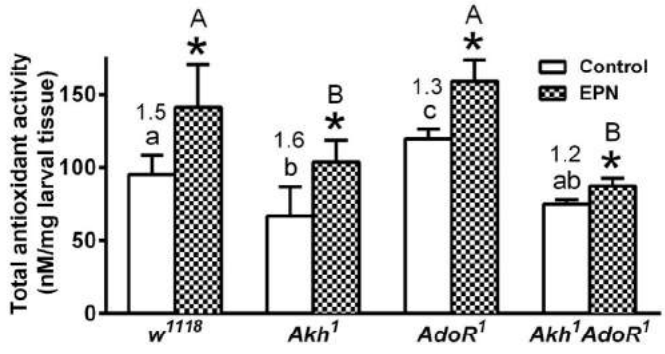


Fig. 7. The effect of entomopathogenic nematode (EPN) *S. carpocapsae* on total anti-oxidant activity in body of various *D. melanogaster* mutants 24 h after the treatment. Statistically significant differences among the mutants at the 5% level evaluated by one-way ANOVA with Tukey's post-test are indicated by different letters (effect of mutation); differences between the experimental groups within the mutant (effect of treatment) at the 5% level evaluated by Student's *t*-test are indicated by asterisks. The numbers above the columns represent fold-difference of total anti-oxidant activity after treatments by *S. carpocapsae* as compared with the controls; n = 5.

significantly post-EPN infection in all groups (Fig. 5). However, infected and uninfected loss-of-function mutants differed only slightly from infected or uninfected w^{1118} larvae in haemolymph lipid levels.

In contrast to haemolymph lipids, EPN infection significantly dropped haemolymph protein levels, with the highest decline in Akh^1 and Akh^1 $AdoR^1$ (2.9- and 2.3-fold, respectively) (Fig. 6). Further, in uninfected groups, Akh^1 had significantly higher haemolymph proteins, while $AdoR^1$ haemolymph protein levels were significantly lower. Among the infected groups the highest level was recorded in w^{1118} larvae.

Both AKH and adenosine appear to be involved in EPN-induced antioxidative stress response (Fig. 7). Prior to infection, Akh^1 and $AdoR^1$ had significantly lower and higher total antioxidative activity than control, respectively, whereas Akh^1 $AdoR^1$ did not differ from control. After EPN infection, total antioxidative activity increased significantly and similarly from un-infected levels across all groups.

3.2 Locomotion

We found that AKH treatment of w^{1118} larvae significantly increased movement distance by 1.6-fold (Fig. 8A), despite a significant reduction in movement velocity by 1.8-fold (Fig. 8B) because their go phase lasted 2.0 times longer than untreated w^{1118} (Fig. 8C). Similarly, AKH-treated

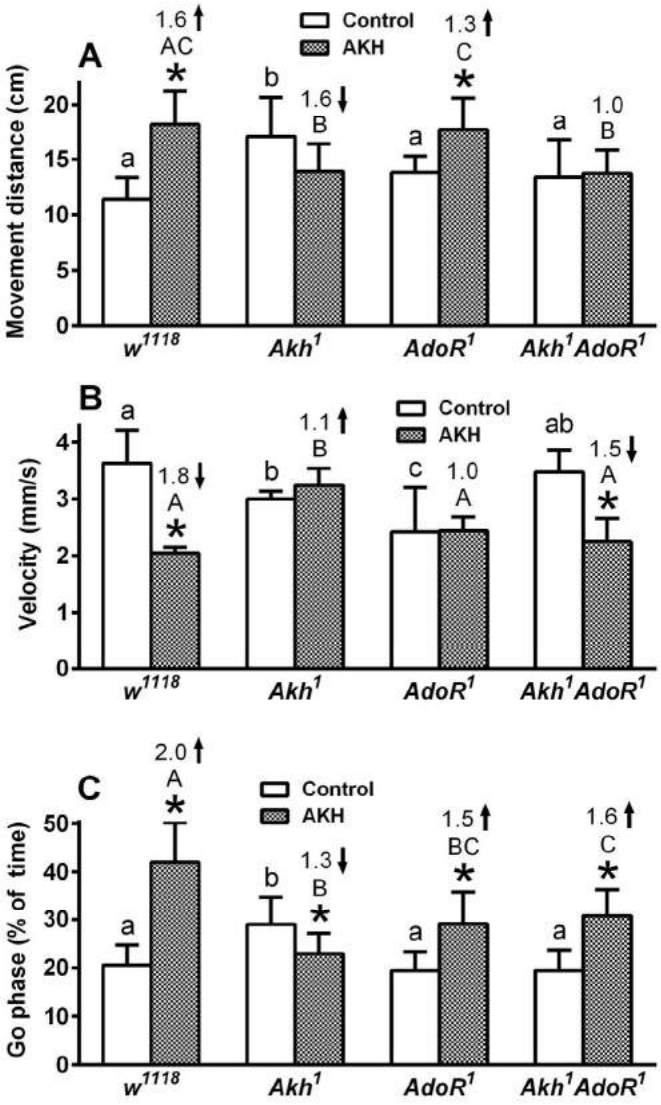


Fig. 8. The effect of Drome-AKH (AKH) on locomotor characteristics of various *D. melanogaster* mutants: movement distance (A), velocity (B) and go phase (C). Statistically significant differences among the mutants at the 5% level evaluated by one-way ANOVA with Tukey's post-test are indicated by different letters (effect of mutation); differences between the experimental groups within the mutant (effect of treatment) at the 5% level evaluated by Student's *t*-test are indicated by asterisks. The numbers above the columns represent fold-difference of the locomotor characteristics after treatments by AKH as compared with the controls. Each column represents 8 parallel measurements with 15 larvae per each.

$AdoR^1$ mutants showed significant increase in movement distance and go phase (1.3 and 1.5-fold, respectively), whereas velocity did not change. Surprisingly, AKH treatment did not influence movement distance or velocity of Akh^1 mutants; go phase duration was significantly inhibited, but the decrease was relatively small, by only 1.3-fold. Finally, AKH treatment significantly reduced velocity and increased go phase in Akh^1 $AdoR^1$ mutants, yielding the same movement distance. A comparison of fruit flies untreated with AKH revealed that movement distance was longer by 1.5 times in Akh^1 larvae than in w^{1118}, a difference accompanied by significant increase in go-phase duration and reduction in velocity (1.4- and 1.3-fold respectively; Fig. 8).

4. Discussion

4.1 Metabolic aspects

Infection by nematobacterial complexes represents a severe stressor for the attacked host insect. Although AKHs are released as part of an insect's generalized defense response to pathogen attack (including EPNs), the hormone's function in activating energy-rich metabolic substrates may occasionally do more harm than good. Our results in the current study support this possibility; we demonstrated that AKH treatment of EPN-infected *Drosophila* larvae exhibited significantly higher mortality than infected larvae not subjected to AKH. Additionally, Akh^1 mutants experienced the lowest mortality post-EPN infection among all fly groups. The current data are in line with our previous study on *P. apterus*, showing that EPN and Pyrap-AKH co-application increased mortality by 2.5-fold compared with mortality from EPN infection alone; likewise, mortality was lower in *AKH* loss-of-function firebug mutants (Ibrahim et al., 2017). Research from other groups further corroborate these results: AKH treatment increased mortality in *Locusta migratoria* infected with entomopathogenic *Metarhizium anisopliae* or *Bacillus megaterium* (Goldsworthy et al., 2005).

Two factors provide a possible explanation for this phenomenon. Firstly, EPN + AKH treatment increased total metabolism in fly larvae as compared with the EPN alone treated larvae (documented by the carbon dioxide production - see Fig. 3). In turn, this elevated metabolism may intensify metabolite turnover, including EPN-released toxins. Thus, these toxins can penetrate cells faster, increasing their negative influence on insect physiological and biochemical functions. And those might be even faster than any defense reactions of the body. Indeed, co-application of AKH and insecticides actually increased the latter's efficacy in multiple insects (Kodrík et al., 2010; Kodrík et al., 2015c; Velki et al., 2011; Plavšin et al., 2015). The second way in which AKH application may increase mortality is through the hormone's ability to mobilize nutrients (Mullen and Goldsworthy, 2006). Nutrient increase in the haemolymph can spur pathogen proliferation, thereby increasing virulence. Supporting this hypothesis, we observed that trehalose was lowest in both infected and uninfected Akh^1 mutants, correlating with their low mortality. Correspondingly, w^{1118} exhibited the highest increase in trehalose post-EPN treatment. These results accord with AKH's reported control of trehalose concentrations in *Drosophila* haemolymph (Kim and Rulifson, 2004; Lee and Park 2004; Isabel et al., 2005).

However, the effect of EPN infection on haemolymph nutrients beside trehalose appears to be more complicated. Lipid concentrations increased uniformly post-EPN treatments in all mutants, suggesting that AKH and adenosine likely do not play a critical role in lipid mobilization. Several previous studies have also reported that AKH has little effect on lipid levels in *Drosophila* haemolymph (Isabel et al. (2005; Gáliková et al., 2015).

Interestingly, uninfected Akh^1 mutants had the highest haemolymph protein concentration, probably due to the known role of AKH in inhibition of protein synthesis (Carlisle and Loughton, 1986; Kodrík and Goldsworthy, 1995). Upon EPN infection, protein content decreased significantly in all tested flies, likely for three non-mutually exclusive reasons. First, EPN-secreted products have high proteolytic activity to allow digestion and subsequent use of host proteins (Toubarro et al., 2009; Jing et al. 2010; Rougon-Cardoso et al., 2016). Second, low haemolymph protein levels may reflect a massive anti-stress response that employs numerous defense proteins. Finally, mutualistic bacteria associated with nematodes establish their population quickly in an infected host and eventually colonize all tissues (Ffrench-Constant et al., 2003). Their presence and metabolism likely contributes to further protein depletion in the haemolymph. Further studies are necessary to provide empirical evidence of these possible explanations.

Despite similarities in function and signalling pathways (Park et al., 2002; Jacobson and Gao, 2009; Žuberová et al., 2010), AKH and adenosine do not appear to cooperate (Žuberová et al., 2010; Zemanová et al., 2016), instead operating in parallel. Our results here confirm the lack of interaction between AKH and adenosine. Moreover, AKH's role in the various processes examined in this study appears to reflect the hormone's primary function, whereas effect of adenosine appears indirect, possibly derived from other functions related to biochemical and physiological homeostasis.

4.2 Oxidative stress

Here, we demonstrated that AKH and, to a lesser extent, adenosine, modulates antioxidative stress responses in EPN-infected *Drosophila*. Our results are thus in line with the existing body of work showing that AKHs in insects (including *Drosophila*) contribute to defense against several reactive oxygen species that cause oxidative stress (paraquat, hydrogen peroxide, tannic acid, Cry 3A toxin, insecticides) (Krishnan and Kodrík, 2012; Kodrík et al., 2015b; Gáliková et al., 2015; Zemanová et al., 2016). Some evidence also exists to show that adenosine is involved in the antioxidative stress response (Zemanová et al., 2016). However, while Akh^1 and Akh^1 $AdoR^1$ mutants have the lowest level of total antioxidant activity (before EPN infection), we also observed slightly higher antioxidant activity in $AdoR^1$ mutant, suggesting an ambiguous role of adenosine in anti-oxidative stress response. We also found indirect evidence that more mechanisms beyond AKH and adenosine must be involved in antioxidant defense because all experimental groups experienced the same pattern of increase in total antioxidant activity after EPN infection. This conclusion is in line with previous work with the same mutants, suggesting complex systems controlling antioxidative stress response in *Drosophila* (Zemanová et al., 2016), including the involvement of *glutathione S-transferase D1* (*GstD1*). In addition, reactive oxygen species (ROS) treatment increased *GstD1* expression in the following order: $w^{1118} > AdoR^1 > Akh^1 > Akh^1$ $AdoR^1$. Thus, while both adenosine and AKH are important for increasing antioxidant-related gene expression, the latter probably plays a more important role; this conclusion is similar to our current findings. Surprisingly, actual GST enzyme activity was unaffected by mutation (in *Akh* and *AdoR*) or ROS treatment. Further, the antioxidant glutathione was significantly lower in untreated Akh^1, $AdoR^1$, and Akh^1 $AdoR^1$ mutant larvae than in untreated w^{1118} controls (Zemanová et al., 2016).

4.3 Locomotor activities

Although AKH control of *Drosophila* locomotion has been previously recorded (Lee and Park 2004; Isabel et al., 2005), in line with numerous data on similar roles (muscle contraction, general locomotion) in other species (O'Shea et al., 1984; Scarborough et al., 1984; Witten et al., 1984; Socha et al., 1999; Kodrík et al., 2000; Lorenz et al., 2004), our current data suggest that this function is less straightforward than previously thought. We showed that AKH application does affect locomotion (especially go phase duration) in w^{1118} *Drosophila* larvae, but when done to Akh^1 mutants, the effect on locomotion was negligible. The possibility that AKH's involvement in locomotion may be more complex has also been suggested in several previous studies. For instance, experiments that ablated AKH-producing cells in the corpora cardiaca of *Drosophila* resulted in significant hypoactivity (Isabel et al., 2005). Furthermore, starved flies without AKH-producing neurons did not exhibit the increased locomotor activity of starved control flies (Lee and Park, 2004). The data suggest that AKH's role in locomotion is to induce foraging behaviour during energy shortage, other studies that did not entirely ablate corpora cardiaca cells, but instead removed AKH production, did not observe changes to *Drosophila* locomotion (Gáliková et al., 2015). Further, no clear effect of adenosine on any of the locomotor activity has been recorded. We recommend that future studies examine in more detail the various players of AKH (and/or adenosine) signalling pathways controlling *Drosophila* locomotion, to obtain a clearer understanding of a complex mechanism.

In conclusion, our study demonstrated involvement of both AKH and adenosine in *Drosophila* anti-stress responses against EPN infection. *Drosophila* larvae experienced significantly higher mortality when AKH was applied with EPN compared with the application of EPN alone. This phenomenon is likely due to the AKH-triggered increase in metabolism and energy rich metabolite levels. The decrease in haemolymph proteins after EPN infection may have occurred due to EPN-associated proteases or might reflect massive involvement of defense proteins in anti-stress response. Moreover, while EPN infection caused oxidative stress, the resultant antioxidative functions involved more mechanisms than AKH and adenosine. Finally, while AKH application affected locomotion, its exact role is not completely clear because *AKH* loss-of-function did not alter locomotion accordingly. Likewise, adenosine exerted very little influence on locomotion. To summarize, AKH exerts an active influence on EPN-induced stress responses, whereas the role of adenosine is smaller and somewhat unclear. Furthermore, the two compounds act separately in *Drosophila* body. All these findings are academically interesting but also contribute data that might be potentially usable in pest management strategies in future: any natural agent able to intensify toxicity of both entomopathogenic organisms and classical insecticides would be undoubtedly interesting for the insect pest control and protection of the environment.

Acknowledgements

This study was supported by grant No. 17-03253S from the Czech Science Foundation, and by projects RVO 60077344 of the Institute of Entomology. FIMTrack table was purchased with funding from Carl Tryggers Foundation CTS16:474. The authors thank Mrs. H. Štěrbová for her technical assistance. The English grammar and stylistics were checked by the Editage Author Services.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.jinsphys.2018.04.002.

References

Arefin, B., Kučerová, L., Dobeš, P., Markus, R., Strnad, H., Wang, Z., Hyršl, P., Žurovec, M., Theopold, U., 2014. Genome-wide transcriptional analysis of drosophila larvae infected by entomopathogenic nematodes shows involvement of complement, recognition and extracellular matrix proteins. J. Innate Immun. 6, 192–204.
Bil, M., Broeckx, V., Landuyt, B., Huybrechts, R., 2014. Differential peptidomics highlights adipokinetic hormone as key player in regulating digestion in anautogenous flesh fly, *Sarcophaga crassipalpis*. Gen. Comp. Endocrinol. 208, 49–56.
Bodláková, K., Jedlička, P., Kodrík, D., 2017. Adipokinetic hormones control amylase activity in the cockroach (*Periplaneta americana*) gut. Insect Sci. 24, 259–269.
Bodláková, K., Beňová, M., Kodrík, D., 2018. The effect of adipokinetic hormones on the activity of digestive enzymes. Entomol Physiol 10.1111/phen.12238.
Carlisle, J., Loughton, B.G., 1986. The inhibition of protein synthesis in *Locusta migratoria* by adipokinetic hormone. J. Insect Physiol. 32, 573–578.
Carroll, N.V., Longley, R.W., Roe, J.H., 1956. The determination of glycogen in liver and muscle by use of anthrone reagent. J. Biol. Chem. 220, 583–593.
Csóka, B., Haskó, G., 2011. Adenosine, inflammation pathways and therapeutic challenges. Jt. Bone Spine 78, 4–6.
Dobeš, P., Wang, Z., Markus, R., Theopold, U., Hyršl, P., 2012. An improved method for nematode infection assays in *Drosophila* larvae. Fly 6, 75–79.
Doležal, T., Doleželová, E., Žurovec, M., Bryant, P.J., 2005. A role for adenosine deaminase in *Drosophila* larval development. PLoS Biol. 3, e201.
Duchaud, E., (25 co-authors), et al., 2003. The genome sequence of the entomopathogenic bacterium *Photorhabdus luminescens*. Nat. Biotechnol. 21, 1307–1313.
Ffrench-Constant, R., Waterfield, N., Daborn, P., Joyce, S., Bennett, H., Au, C., Dowling, A., Boundy, S., Reynolds, S., Clarke, D., 2003. *Photorhabdus*: towards a functional genomic analysis of a symbiont and pathogen. FEMS Microbiol. Rev. 26, 433–456.
Gäde, G., Auerswald, L., 2003. Mode of action of neuropeptides from the adipokinetic hormone family. Gen. Comp. Endocrinol. 132, 10–20.
Gäde, G., Hoffmann, K.H., Spring, J.H., 1997. Hormonal regulation in insects: facts, gaps, and future directions. Physiol. Rev. 77, 963–1032.
Gáliková, M., Diesner, M., Klepsatel, P., Hehlert, P., Xu, Y., Bickmeyer, I., Predel, R., Kühnlein, R.P., 2015. Energy homeostasis control in *Drosophila* adipokinetic hormone mutants. Genetics 201, 665–683.
Goldsworthy, G.J., Opoku-Ware, K., Mullen, L.M., 2002. Adipokinetic hormone enhances laminarin and bacterial lipopolysaccharide-induced activation of the prophenoloxidase cascade in the African migratory locust, *Locusta migratoria*. J. Insect Physiol. 48, 601–608.
Goldsworthy, G.J., Chandrakant, S., Opoku-Ware, K., 2003. Adipokinetic hormone enhances nodule formation and phenoloxidase activation in adult locusts injected with bacterial lipopolysaccharide. J. Insect Physiol. 49, 795–803.
Goldsworthy, G.J., Opoku-Ware, K., Mullen, L.M., 2005. Adipokinetic hormone and the immune responses of locusts to infection. Ann. N. Y. Acad. Sci. 1040, 106–113.
Hyršl, P., Dobeš, P., Wang, Z., Hauling, T., Wilhelmsson, C., Theopold, U., 2011. Clotting factors and eicosanoids protect against nematode infections. J. Innate Immun. 2, 65–70.
Ibrahim, E., Hejníková, M., Shaik, H.A., Doležel, D., Kodrík, D., 2017. Adipokinetic hormone activities in insect body infected by entomopathogenic nematode. J. Insect Physiol. 98, 347–355.
Isabel, G., Martin, J.R., Chidami, S., Veenstra, J.A., Rosay, P., 2005. AKH-producing neuroendocrine cell ablation decreases trehalose and induces behavioural changes in *Drosophila*. Am. J. Physiol. Regul. Integr. Comp. Physiol. 288, 531–538.
Jacobson, K.A., Gao, Z.G., 2009. Adenosine. In: Malenka, R.C. (Ed.), Intercellular Communication in the Nervous System. Elsevier, Boston, USA, pp. 627–638.
Jančařík̆ová, G., Houser, J., Dobeš, P., Demo, G., Hyršl, P., Wimmerová, M., 2017. Characterization of novel bangle lectin from *Photorhabdus asymbiotica* with dual sugar-binding specificity and its effect on host immunity. PLoS Pathog. 13, e1006564.
Jing, Y., Toubarro, D., Hao, Y., Simões, N., 2010. Cloning, characterisation and heterologous expression of an astacin metalloprotease, Sc-AST, from the entomoparasitic nematode *Steinernema carpocapsae*. Mol. Biochem. Parasit. 174, 101–108.
Kim, S.K., Rulifson, E.J., 2004. Conserved mechanisms of glucose sensing and regulation by *Drosophila* corpora cardiaca cells. Nature 431, 316–320.
Kodrík, D., 2008. Adipokinetic hormone functions that are not associated with insect flight. Physiol. Entomol. 33, 171–180.
Kodrík, D., Goldsworthy, G.J., 1995. Inhibition of RNA synthesis by adipokinetic hormones and brain factor(s) in adult fat body of *Locusta migratoria*. J. Insect Physiol. 41, 127–133.
Kodrík, D., Socha, R., Šimek, P., Zemek, R., Goldsworthy, G.J., 2000. A new member of the AKH/RPCH family that stimulates locomotory activity in the firebug, *Pyrrhocoris apterus* (Heteroptera). Insect Biochem. Mol. Biol. 30, 489–498.
Kodrík, D., Šimek, P., Lepša, L., Socha, R., 2002. Identification of the cockroach neuropeptide Pea-CAH-II as a second adipokinetic hormone in the firebug *Pyrrhocoris apterus*. Peptides 23, 585–587.
Kodrík, D., Socha, R., Syrová, Z., 2003. Developmental and diel changes of adipokinetic hormone in CNS and haemolymph of the flightless wing-polymorphic bug, *Pyrrhocoris apterus*. J. Insect Physiol. 49, 53–56.
Kodrík, D., Krishnan, N., Habuštová, O., 2007. Is the titer of adipokinetic peptides in *Leptinotarsa decemlineata* fed on genetically modified potatoes increased by oxidative stress? Peptides 28, 974–980.
Kodrík, D., Bártů, I., Socha, R., 2010. Adipokinetic hormone (Pyrap-AKH) enhances the effect of a pyrethroid insecticide against the firebug *Pyrrhocoris apterus*. Pest Manag. Sci. 66, 425–431.
Kodrík, D., Vinokurov, K., Tomčala, A., Socha, R., 2012. The effect of adipokinetic hormone on midgut characteristics in *Pyrrhocoris apterus* L. (Heteroptera). J. Insect Physiol. 58, 194–204.
Kodrík, D., Bednářová, A., Zemanová, M., Krishnan, N., 2015a. Hormonal regulation of response to oxidative stress in insects - an update. Int. J. Mol. Sci. 16, 25788–25816.
Kodrík, D., Plavšin, I., Velki, M., Stašková, T., 2015b. Enhancement of insecticide efficacy by adipokinetic hormones. In: Montgomery, J. (Ed.), Insecticides: Occurrence, Global Threats and Ecological Impact. Nova Science Publishers Inc, New York, pp. 77–91.
Kodrík, D., Stašková, T., Jedličková, V., Weyda, F., Závodská, R., Pflegerová, J., 2015c. Molecular characterization, tissue distribution, and ultrastructural localization of adipokinetic hormones in the CNS of the firebug *Pyrrhocoris apterus* (Heteroptera, Insecta). Gen. Comp. Endocrinol. 210, 1–11.
Krishnan, N., Kodrík, D., 2012. Endocrine control of oxidative stress in insects. In: Farooqui, T., Farooqui, A.A. (Eds.), Oxidative Stress in Vertebrates and Invertebrates: Molecular Aspects of Cell Signaling. Wiley-Blackwell, New Jersey, pp. 261–270.
Kunc, M., Arefin, B., Hyršl, P., Theopold, U., 2017. Monitoring the effect of pathogenic nematodes on locomotion of *Drosophila* larvae. Fly 11, 208–217.
Lee, G., Park, J.H., 2004. Haemolymph sugar homeostasis and starvation-induced hyperactivity affected by genetic manipulations of the adipokinetic hormone-encoding gene in *Drosophila melanogaster*. Genetics 167, 311–323.
Lorenz, M.W., Gäde, G., 2009. Hormonal regulation of energy metabolism in insects as a driving force for performance. Integr. Comp. Biol. 49, 380–392.
Lorenz, M.W., Zemek, R., Kodrík, D., Socha, R., 2004. Lipid mobilisation and locomotor stimulation in *Gryllus bimaculatus* (de Geer) (Ensifera, Gryllidae) by topically applied adipokinetic hormone. Physiol. Entomol. 29, 146–151.
Lu, D., Macchietto, M., Chang, D., Barros, M.M., Baldwin, J., Mortazavi, A., Dillman, A.R., 2017. Activated entomopathogenic nematode infective juveniles release lethal venom proteins. PLoS Pathog. 13 (4), e1006302.
Milde, J.J., Ziegler, R., Wallstein, M., 1995. Adipokinetic hormone stimulates neurons in the insect central nervous system. J. Exp. Biol. 198, 1307–1311.
Mullen, L.M., Goldsworthy, G.J., 2006. Immune responses of locusts to challenge with the pathogenic fungus *Metarhizium* or high doses of laminarin. J. Insect Physiol. 52, 389–398.
O'Shea. M., Witten. J., Schaffer. M., 1984. Isolation and characterization of two myoactive neuropetides: further evidence for an invertebrate peptide family. J. Neurosci. 4, 521–529.
Park, Y., Kim, Y.J., Adams, M.E., 2002. Identification of G protein-coupled receptors for *Drosophila* PRXamide peptides, CCAP, corazonin, and AKH supports a theory of ligand-receptor coevolution. Proc. Nat. Acad. Sci. U.S.A. 99, 11423–11428.
Plavšin, I., Stašková, T., Šerý, M., Smýkal, V., Hackenberger, H.K., Kodrík, D., 2015.

Hormonal enhancement of insecticide efficacy in *Tribolium castaneum*: oxidative stress and metabolic aspects. Com. Biochem. Physiol. C170, 19–27.
Risse, B., Berh, D., Otto, N., Klämbt, C., Jiang, X., 2017. FIMTrack: an open source tracking and locomotion analysis software for small animals. PLOS Comput. Biol. 13 (5), e1005530.
Rougon-Cardoso, A., Flores-Ponce, M., Ramos-Aboites, H.E., Martínez-Guerrero, C.E., Hao, Y.J., Cunha, L., Rodríguez-Martínez, J.A., Ovando-Vázquez, C., Bermúdez-Barrientos, J.R., Abreu-Goodger, C., Chavarría-Hernández, N., Simões, N., Montiel, R., 2016. The genome, transcriptome, and proteome of the nematode *Steinernema carpocapsae*: evolutionary signatures of a pathogenic lifestyle. Sci. Rep. 6, 37536.
Sajwan, S., Sidorov, R., Stašková, T., Žaloudíková, A., Takasu, J., Kodrík, D., Žurovec, M., 2015. Targeted mutagenesis and functional analysis of adipokinetic hormone-encoding gene in *Drosophila*. Insect Biochem. Mol. Biol. 61, 79–86.
Scarborough, R.M., Jamieson, G.C., Kramer Kalisz, S.J., McEnroe, G.A., Miller, C.A., Schooled, D.A., 1984. Isolation and primary structure of two peptides with cardioacceleratory and hyperglycaemic activity from the corpora cardiaca of *Periplaneta americana*. Proc. Nat. Acad. Sci. U.S.A. 81, 5575–5579.
Schaffer, M.H., Noyes, B.E., Slaughter, C.A., Thorne, G.C., Gaskell, S.J., 1990. The fruitfly *Drosophila melanogaster* contains a novel charged adipokinetic-hormone-family peptide. Biochem. J. 269, 315–320.
Simões, N., Caldas, C., Rosa, J.S., Bonifassi, E., Laumond, C., 2000. Pathogenicity caused by high virulent and low virulent strains of *Steinernema carpocapsae* to *Galleria mellonella*. J. Invertebr. Pathol. 75, 47–54.
Socha, R., Kodrík, D., Zemek, R., 1999. Adipokinetic hormone stimulates insect locomotor activity. Naturwissenschaften 88, 85–86.
Socha, R., Kodrík, D., Šimek, P., Patočková, M., 2004. The kind of AKH-mobilized energy substrates in insects can be predicted without a knowledge of the hormone structure. Eur. J. Entomol. 101, 29–35.
Stoscheck, C.M., 1990. Quantitation of proteins. In: In: Deutscher, M.P. (Ed.), Methods in Enzymology, vol. 182. Academic Press, London, pp. 50–68.
Toubarro, D., Lucena-Robles, M., Nascimento, G., Costa, G., Montiel, R., Coelho, A.V., Simões, N., 2009. An apoptosis-inducing serine protease secreted by the entomopathogenic nematode *Steinernema carpocapsae*. Int. J. Parasitol. 29, 1319–1330.
Velki, M., Kodrík, D., Večeřa, J., Hackenberger, B.K., Socha, R., 2011. Oxidative stress elicited by insecticides: a role for the adipokinetic hormone. Gen. Comp. Endocrinol. 172, 77–84.
Wang, Z., Wilhelmsson, C., Hyršl, P., Loof, T.G., Dobeš, P., Klupp, M., Loseva, O., Mörgelin, M., Iklé, J., Cripps, R.M., Herwald, H., Theopold, U., 2010. Pathogen entrapment by transglutaminase-A conserved early innate immune mechanism. PLoS. Pathog. 6, 1–9.
Wicher, D., Agricola, H.J., Sohler, S., Gundel, M., Heinemann, S.H., Wollweber, L., Stengl, M., Derst, C., 2006. Differential receptor activation by cockroach adipokinetic hormones produces differential effects on ion currents, neuronal activity, and locomotion. J. Neurophysiol. 95, 2314–2325.
Withers, P.C., 1977. Measurement of V_{O2}, V_{CO2} and evaporative water loss with a flow-through mask. J. Appl. Physiol. 42, 120–123.
Witten, J.L., Schaffer, M.H., O'Shea, M., Cook, J.C., Hemling, M.E., Rinehart Jr., K.L., 1984. Structures of two cockroach neuropeptides assigned by fast atom bombardment mass spectrometry. Biochem. Biophys. Res. Commun. 124, 350–358.
Zemanová, M., Stašková, T., Kodrík, D., 2016. Role of adipokinetic hormone and adenosine in the anti-stress response in *Drosophila melanogaster*. J. Insect Physiol. 91–92, 39–47.
Zöllner, N., Kirsch, K., 1962. Über die quantitative Bestimmung von Lipoide (Mikromethode) mittels der vielen natürlichen Lipoiden (allen bekannten Plasmalipoiden) gemeinsamen Sulfo-phospho-vanillin-Reaktion. Z Ges. Exp. Medizin. 135, 545–561.
Žuberová, M., Fencková, M., Šimek, P., Janečková, L., Doležal, T., 2010. Increased extracellular adenosine in *Drosophila* that are deficient in adenosine deaminase activates a release of energy stores leading to wasting and death. Dis. Model. Mech. 3, 773–784.

5.3. Manuscript

Kodrík D., Ibrahim E., Gautam U. K., Frydrychová R., Bednářová A., Krištůfek V., and Jedlička P. Changes in vitellogenin expression caused by nematodal and fungal infections in insects. J. *Exp. Biol.* Submitted manuscript.

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

Dalibor Kodrík[1,2,*], Emad Ibrahim[1,2,5], Umesh K. Gautam[1,2], Radmila Čapková Frydrychová[1], Andrea Bednářová[2], Václav Krištůfek[3] and Pavel Jedlička[4]

[1]Institute of Entomology, Biology Centre, CAS, Branišovská 31, 370 05 České Budějovice, Czech Republic
[2]Faculty of Science, University of South Bohemia, Branišovská 31, 370 05 České Budějovice, Czech Republic
[3]Institute of Soil Biology, Biology Centre, CAS, Branišovská 31, 370 05 České Budějovice, Czech Republic
[4]Institute of Biophysics, CAS, Královopolská 135, 612 65 Brno, Czech Republic
[5]Faculty of Agriculture, University of Cairo, Giza, Egypt

* Author for correspondence: Institute of Entomology, Biology Centre, CAS, Branišovská 31, 370 05 České Budějovice, Czech Republic
E-mail address: kodrik@entu.cas.cz
ID D.K., https://orcid.org/0000-0001-6109-1979

Summary statement

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

Abstract

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

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

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

INTRODUCTION

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

molecule directly in the fat body (Carlisle and Loughton, 1986). Besides this, Vg production is controlled by the nutrient levels and mating status (Chapman, 1998).

Vgs are typically present in egg-laying females. However, low levels of Vgs have been identified in males of several insect species, including the firebug *Pyrrhocoris apterus* (Němec et al., 1993). Several recent studies suggested that Vgs play an important role not only in the reproduction, but also in other aspects of insect biology, such as caste differentiation process in social insects, wound healing, protection against oxidative stress, immunity, and life span regulation (Havukainen et al., 2013; Singh et al., 2013; Salmela et al., 2015; Salmela and Sundstrom, 2017; Park et al., 2018). Studies in the silkworm and honeybees reported strong antibacterial activity of Vg against gram-positive and gram-negative bacteria (Singh et al., 2013), showing that Vg bound to bacterial cells and destroyed them. Vg was active even against *Paenibacilus larvae,* a gram-positive bacterium infesting young honeybee larvae and causing a disease called the American foulbrood, probably the deadliest bee brood disease worldwide (Salmela et al., 2015). In addition, it has been reported that infection of honey bee larvae by the spores of the microsporidium *Nosema ceranae* significantly up-regulated Vg expression in workers (BenVau and Nieh, 2017; Sinpoo et al., 2018), and that bee Vg interacted with the cell wall of the entomopathogenic fungus *Beauveria bassiana*, eliciting membrane disruption and permeabilization. Furthermore, Vg appears to induce trans-generational immune priming in bee queens, enhancing immunity in their offspring by transporting pathogen-associated pattern molecules, which are attached to Vg, into the eggs within queen ovaries (Sadd et al., 2005; Salmela et al., 2015).

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

workers and queens, in which Vg is synthesized in high levels. However, exact mechanisms of the anti-oxidative effects of Vg and hormones in insect body remain unclear.

In the present study, we sought to examine Vg role in the defense against two different entomopathogens: the nematode *Steinernema carpocapsae* and the fungus *Isaria fumosorosea*. The nematode *S. carpocapsae* carries symbiotic *Xenorhabdus* spp. bacteria that are toxic for insects (Simões et al., 2000; Duchaud et al., 2003), and the nematobacterial complex represents an efficient tool for insect killing commonly used for insect pest control (Ehlers, 2003; Inman et al., 2012). Similarly, the fungus *I. fumosorosea,* harbored by the horse chestnut leaf miner *Cameraria ohridella,* plays a significant role in the biological control of many insect species (Zimmermann, 2008).The main aim of the present study was (1) to examine changes in Vg gene and protein expression upon the infection with entomopathogenic nematode (EPN) and entomopathogenic fungus (EPF); (2) to elucidate the role of Vg in insect body infected by EPN, and (3) to determine whether Vg-mediated defense reaction in response to EPF, similar to that observed in honey bees or silkworms (see above), is a common defense mechanism in insects. In addition (4), we sought to explain the role of Vg in insect males.

MATERIAL AND METHODS

Experimental insects

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

Entomopathogenic nematode *Steinernema carpocapsae* and the insect treatment

The nematodes *S. carpocapsae* originating from Russia (strain NCR), St. Petersburg were obtained by courtesy of Dr. Z. Mráček (Institute of Entomology, České Budějovice). They were reared under laboratory conditions using the last

larval instar of *Galleria mellonella* (Lepidoptera, Insecta) as a host. The emerging infective juveniles were harvested and subsequently stored in water at 4°C for 30 days. Their viability was confirmed under a microscope before experiments.

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

Entomopathogenic fungus *Isaria fumosorosea* and the insect treatment

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

RNA and cDNA preparation, primers

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

- *RNA isolation and cDNA synthesis.* The total RNA was extracted using TRI Reagent® (Sigma-Aldrich) following the manufacturer's protocol. RNA isolates were treated with RQ1 RNase-Free DNase (Promega) to remove traces of contaminant DNA. The cDNA template was prepared using the SuperScript® III First-Strand Synthesis System for RT-PCR (Invitrogen by Life Technologies) on 2 µg of the corresponding total RNA with random hexamers.

Quantification of *Vg* gene expression

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

Gel electrophoresis and vitellogenin quantification

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

Western blotting

After SDS-PAGE the separated proteins were blotted onto nitrocellulose membrane according to Towbin et al. (1979). Specific antibody against *P. apterus* Vg (1: 1000; Socha et al., 1991) was used in the procedure including the secondary antibody Goat/HRP (1:1000; goat anti rabbit labelled with horse radish peroxidase; Sigma-Aldrich). For visualization the Novex® ECL HRP chemiluminescent substrate reagent kit (Invitrogen) in A and B solutions 1:1 was used. The developed color was documented using Intelligent Dark Box (LAS 3000, Fujifilm).

Vg isolation and its antimicrobial activity

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

the samples measured at 562 nm to µg proteins. Thereafter the samples were stored at -20° C until needed.

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

Mortality test

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

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

Data presentation and statistical analysis

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

RESULTS

Steinernema carpocapsae infection

The first series of experiments focused on measuring *Vg* transcript levels during nematobacterial infection of the fat body of male *P. apterus*. Infection of males with the EPN *S. carpocapsae* resulted in a 7.7-fold increase of *Vg* transcript level on the next day post infection (Fig. 1). Vg protein level in hemolymph was also increased 1.4-fold, as visualized by PAGE (Fig. 2A, C). These observations were then verified using western blot with an anti-Vg antibody (Fig. 2B). Interestingly, the anti-Vg antibody used in western blot positively recognized not only the main Vg band (about 180 kDa), but also two smaller bands of 65 and 24 kDa, which might be degradation products of EPN toxic actions in the body of *P. apterus* males.

As expected, *Vg* gene expression level was substantially higher in the fat bodies of females than in those of males (Fig. 3). *Vg* transcript levels in females continuously increased during the first 5 days of development (Fig. 3), i.e., the critical period for egg formation. Application of EPN radically reduced the level of Vg transcripts in 2–5-day old females on the next day (Fig. 3); the extent of inhibition ranged from about 9- (in 4-day-old) to about 150-fold (in 3-day-old females). A similar trend was observed with Vg protein levels in hemolymph, although the differences were not so profound (Fig. 4): 12-fold change (in 3-day old females) was recorded as the maximal one (Fig. 4C). These results were confirmed by immunoanalysis using western blotting (Fig. 4B). In contrast to the reaction observed in males, the antibody recognized only the 180 kDa Vg band in female hemolymph. This suggests that male and female bodies reacted differently to EPN infection.

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

maybe due to a higher level of Vg in the female body. Further, using the disc diffusion method, we tested the antimicrobial effect of Vg on growth of the bacterium *Xenorhabdus* spp. isolated from *S. carpocapsae* body (Fig. 5B). We found that Vg inhibited *Xenorhabdus* growth; the inhibition was 2.5-fold more effective than in controls.

Isaria fumosorosea infection

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

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

DISCUSSION

Pathogenic organisms elicit severe stress in the host body, which results in the disruption of functional homeostasis (Ivanovič and Jankovič-Hladni, 1991) and activation of characteristic defense response to eliminate or at least to reduce the impact of the stress on the organism. This response occurs at both organismal and cellular levels (Hightower, 1991), and may include both humoral and cellular defenses. The humoral response includes production of various compounds, such as eicosanoids, phenoloxidases, proteinases, proteinase inhibitors, and a wide selection of antimicrobial peptides and proteins (Jiang, 2008; Beckage, 2008). The list of the protective compounds includes also Vgs, whose involvement into insect

defense system has recently been described in several insect species (Havukainen et al., 2013; Singh et al., 2013; Salmela et al., 2015; Salmela and Sundstrom, 2017; Park et al., 2018). We have found in this study that in the firebug *P. apterus*, Vg is probably also involved into the defense reaction against the entomopathogenic nematode *S. carpocapsae* and, partially, against the fungus *I. fumosorosea*.

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

We have found in this study that the nematobacterial complex of *S. carpocapsae* and *Xenorhabdus* spp. affected Vg characteristics in both male and female *P. apterus*. In males, a significant stimulatory effect of the infection was noted on both *Vg* transcript level in the fat body and on Vg protein level in hemolymph. To the best of our knowledge, this is the first report about a stimulatory effect of the nematobacterial complex on Vg production in infected insects. However, at this point, we cannot state, based on our results, whether the effect was primarily elicited by the nematode, its symbiotic bacteria *Xenorhabdus* spp., or by the combined effect of the exposure to both of them. However, it is known that both organisms are insect pathogens (Herbert and Goodrich-Blair, 2007; Waterfield et al., 2009). Further, it looks that interaction of male Vg with EPN results not only in the stimulation of Vg synthesis, but also in the degradation of Vg molecules. Immunoblotting results clearly showed at least two products that positively reacted with the anti-Vg antibody with molecular weights (24 and 65 kDa) well below the molecular mass of 180 kDa of the intact protein. Thus, the real

increase of the Vg level in infected male hemolymph might be higher than that actually measured, and could be closer to the results obtained for Vg transcripts. All these results suggest an active role of Vg against EPN infection in the male body. It is obvious that Vg plays a key role as an irreplaceable component of yolk in eggs developing in the female body, however Vg role in immunity, perhaps secondary, seems to be important as well. Evidence about the presence of Vg in male body has been rather scarce, but Vgs have been identified in *P. apterus* (Němec et al., 1993), *Apis mellifera* (Vilar and Grozinger, 2017), and *Bombus terrestris* (Jedlička et al., 2016) males. Nevertheless, a comprehensive understanding of Vg role in male insects is missing.

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

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

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

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

2B and 7B), because products of Vg degradation observed following EPN infection were completely absent after the exposure to EPF. This suggests different responses of the male body to these infections: the involvement of Vg in the defense reaction is apparently less intensive in the case of EPF infection. It remains to be determined whether any other defense systems are involved into responses to infections with EPN and EPF. In contrast, the response of firebug females to the EPF infection was quite similar to that elicited by EPN: both mRNA and protein Vg levels were significantly down-regulated. Similar reaction was recorded in the whitefly *Bemisia tabaci*, in which both *I. fumosorosea* mycelium (*in vivo*) and fungal extracts (*in vitro*) showed a decrease in Vg level, and corresponding damage of the ovaries (Gao et al., 2017). Possible reasons for this phenomenon have already been discussed in the previous paragraph. In addition, active involvement of Vg into the defense reaction of *A. mellifera* against the entomopathogenic fungus *B. basiana* was recently described by Park et al. (2018), who demonstrated that Vg behaves as a typical anti-microbial peptide. *A. mellifera* Vg bound to *B. basiana* cells and induced structural damage of the cell wall leading to anti-microbial activity against the fungus. Interestingly, Vg is also present in the venom of the honey bee and some other hymenopterans (Blank et al., 2013; Park et al., 2018), where it probably serves as an allergen that intensifies venom efficacy by causing allergic reaction in the stung tissue. Vg is also expressed in the brain of several hymenopteran species, where it controls various processes, including aging (Munch et al., 2015; Lockett et al., 2016; Gospocic et al., 2017). Furthermore, in bees, drone reproductive behavior is modulated by Vg, the expression of which is stimulated by queen pheromones (Villar and Gronzier, 2017).

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

Acknowledgements

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

Competing interests

The authors declare no competing or financial interests.

Author contributions

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

Funding

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

References

Arefin, B., Kučerová, L., Dobeš, P., Markus, R., Strnad, H., Wang, Z., Hyršl, P., Zurovec, M. and Theopold, U. (2014). Genome-wide transcriptional analysis of *Drosophila* larvae infected by entomopathogenic nematodes shows involvement of complement, recognition and extracellular matrix proteins. *J. Inn. Immun.* **6**, 192-204.

Ali, S., Huang, Z. and Ren, S. (2010). Production of cuticle degrading enzymes by *Isaria fumosorosea* and their evaluation as a biocontrol agent against diamondback moth. *J. Pest. Sci.* **83**, 361–370.

Beckage, N. E. (2008). *Insect Immunology*. San Diego, USA: Academic Press Elsevier,

Beckman, K.B. and Ames, B.N. (1998). The free radical theory of aging matures. *Physiol. Rev.* **78**, 547-581.

Bednářová A., Kodrík D., Krishnan N. (2013) Adipokinetic hormone exerts its anti-oxidative effects using a conserved signal- transduction mechanism involving both PKC and cAMP by mobilizing extra- and intracellular Ca^{2+} stores. *Comp. Biochem. Physiol. C* **158**, 142-149.

BenVau, L.R. and Nieh, J. C. (2017). Larval honey bees infected with *Nosema ceranae* have increased vitellogenin titers as young adults. *Sci. Rep.* **7**, article number 14144.

Blank, S., Seismann, H., McIntyre, M., Ollert, M., Wolf, S., Bantleon, F.I. and Spillner, E. (2013). Vitellogenins are new high molecular weight components and allergens (Api m 12 and Ves v. 6) of *Apis mellifera* and *Vespula vulgaris* venom. *PLoS One* **8**, e62009.

Carlisle, J. and Loughton, B.G. (1986). The inhibition of protein synthesis in *Locusta migratoria* by adipokinetic hormone. *J. Insect Physiol.* **32**, 573-578.

Chapman, R.F. (1998). *The Insects, Structure and Function*. Cambridge, UK: Cambridge University Press.

Duchaud, E., Rusniok, C., Frangeul, L., Buchrieser, C., Givaudan, A., Taourit, S., Bocs, S., Boursaux-Eude, C., Chandler M. and Charles, J.F. et al. (2003). The genome sequence of the entomopathogenic bacterium *Photorhabdus luminescens*. *Nat. Biotechnol.* **21**, 1307-1313.

Ehlers, R.U. (2003). Entomopathogenic nematodes in the European biocontrol market. *Commun. Agric. Appl. Biol. Sci.* **68**, 3-16.

Fridovich, I. (1978). The biology of oxygen radicals. *Science* **201**, 875–880.

Gao, T.N., Wang, Z.L., Huang, Y., Keyhani, N.O. and Huang, Z. (2017). Lack of resistance development in *Bemisia tabaci* to *Isaria fumosorosea* after multiple generations of selection. *Sci. Rep.* **7**, article number 42727.

Gospocic, J., Shields, E.J., Glastad, K.M., Lin, Y., Penick, C.C., Hua Yan, H., Mikheyev, A.S., Linksvayer, T.A., Garcia, B.A., Berger, S.L., Liebig, J., Reinberg, D. and Bonasio, R. (2017). The neuropeptide corazonin controls social behavior and caste identity in ants. *Cell* **170**, 748–759.

Hajek, A.E. and Leger, R.J.S. (1994). Interactions between fungal pathogens and insect hosts. In *Annual Review of Entomology* (ed. T.E. Mittler, F.J. Radovsky and V.H. Resh), pp. 293-322. Palo Alto, USA: Annual Reviews, Inc.

Harlow, E. and Lane, D. (1988). *Antibodies. A laboratory manual*. Cold Spring Harbor, USA: Cold Spring Harbor Laboratory.

Havukainen, H., Münch, D., Baumann, A., Zhong, S., Halskau, Ø., Krogsgaard, M. and Amdam, G.V. (2013). Vitellogenin recognizes cell damage through membrane binding and shields living cells from reactive oxygen species. *J. Biol. Chem.* **288**, 28369-28381.

Herbert, E.E. and Goodrich-Blair, H. (2007). Friend and foe: the two faces of *Xenorhabdus nematophila*. *Nat. Rev. Microbiol.* **5**, 634–646.

Hightower, L.E. (1991). Heat-shock, stress proteins, chaperones, and proteotoxicity. *Cell* **66**, 191-197.

Huang, L., Lu, M., Han, G., Dua, Z. and Wang, K. (2016). Sublethal effects of chlorantraniliprole on development, reproduction and vitellogenin gene (CsVg) expression in the rice stem borer, *Chilo suppressalis*. *Pest Manag. Sci.* **72**, 2280–2286

Hyršl, P., Dobeš, P., Wang, Z., Hauling, T., Wilhelmsson, C. and Theopold, U. (2011). Clotting factors and eicosanoids protect against nematode infections. *J. Innate Immun.* **2**, 65-70.

Ibrahim, E., Hejníková, M., Shaik, H.A., Doležel, D. and Kodrík, D. (2017). Adipokinetic hormone activities in insect body infected by entomopathogenic nematode. *J. Insect Physiol.* **98**, 347-355.

Inman F.L., Singh S. and Holmes, L.D. (2012). Mass production of the beneficial nematode *Heterorhabditis bacteriophora* and its bacterial symbiont *Photorhabdus luminescens*. *Indian J. Microbiol.* **52**, 316-324.

Ivanović, J. and Jankovic-Hladni, M. (1991). *Hormones and Metabolism in Insect Stress*. Boca Raton, USA: CRC Press.

Jedlička, P., Ernst, U.R., Votavová, A., Hanus, R. and Valterová, I. (2016). Gene expression dynamics in major endocrine regulatory pathways along the transition from solitary to social life in a bumblebee, *Bombus terrestris*. *Front. Physiol*. 7, Article Number: 574

Jiang, H. (2008). The biochemical basis of antimicrobial responses in *Manduca sexta*. *Insect Sci*. **15**, 53–66.

Kodrík, D. (2008). Adipokinetic hormone functions that are not associated with insect flight. *Physiol. Entomol.* **33**, 171-180

Kodrík, D., Bednářová, A., Zemanová, M. and Krishnan, N. (2015). Hormonal regulation of response to oxidative stress in insects - an update. *Int. J. Mol. Sci.* **16**, 25788-25816.

Krishnan, N., Večeřa, J., Kodrík, D. and Sehnal F. (2007). 20-hydroxyecdysone prevents oxidative stress damage in adult *Pyrrhocoris apterus*. *Arch. Insect Biochem. Physiol*. **65**, 114-124.

Laemmli, U.K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680-685.

Lockett, G.A., Almond, E.J., Huggins, T.J., Parker, J.D. and Bourke, A.F.G. (2016). Gene expression differences in relation to age and social environment in queen and worker bumble bees. *Exp. Gerontol*. **77**, 52–61.

Luangsa-Ard, J.J., Berkaew, P., Ridkaew, R., Hywel-Jones, N.L. and Isaka, M. (2009). A beauvericin hot spot in the genus *Isaria*. *Mycol. Res*. **113**, 1389–1395.

Mahar, A.N., Munir, M., Elawad, S., Gowen, S.R., Hague, N.G.M. (2005). Pathogenicity of bacterium, *Xenorhabdus nematophila* isolated from entomopathogenic nematode (*Steinernema carpocapsae*) and its secretion against *Galleria mellonella* larvae. *J. Zhejiang Univ.-Sci. B*, **6**, 457–463.

Moshitzky, P. and Applebaum, S.W. (1990). The role of adipokinetic hormone in the control of vitellogenin in locusts. *Insect Biochem*. **20**, 319-323.

Munch, D., Ihle, K.E., Salmela, H. and Amdam, G.V. (2015).Vitellogenin in the honey bee brain: Atypical localization of a reproductive protein that promotes longevity. *Exp. Gerontol*. **71**, 103-108.

Němec, V., Kodrík, D., Matolín, S. and Laufer H. (1993). Juvenile hormone effects of retinoic acid in insect metamorphosis, embryogenesis and reproduction. *J. Insect Physiol*. **39**, 1083-1093.

Park, H.G., Lee, S.K., Kim, B.Y., Yoon, H.J., Choi, Y.S., Lee, K.Y., Wan, H., Li, J. and Jin, B.R. (2018). Honeybee (*Apis cerana*) vitellogenin acts as antimicrobial and antioxidant agent in the body and venom. *Dev. Comp. Immunol*. **85**, 51-60.

Prenerová, E., Zemek, R., Weyda, F. and Volter L. (2009). Strain of entomopathogenic fungus *Isaria fumosorosea* CCM 8367 (CCEFO.011.PFR) and the method of controlling insect and mite pests. *Patent No. 300701*. UPV 2008-394.

Sadd, B.M., Kleinlogel, Y., Schmid-Hempel, R. and Schmid-Hempel, P. (2005). Trans-generational immune priming in a social insect. *Biol. Lett*. **1**, 386-388.

Salmela, H. and Sundstrom, L. (2017). Vitellogenin in inflammation and immunity in social insects. *Inflamm. Cell Signal.* **4**, e1506.

Salmela, H., Amdam, G.V. and Freitak, D. (2015). Transfer of immunity from mother to offspring is mediated via egg-yolk protein vitellogenin. *PLoS Pathog.* **11**, e1005015.

Seehuus, S.C., Norberg, K., Gimsa, U., Krekling, T. and Amdam, G.V. (2006) Reproductive protein protects functionally sterile honey bee workers from oxidative stress. *Proc. Natl. Acad. Sci. USA* **103**, 962-967.

Simões, N., Caldas, C., Rosa, J.S., Bonifassi, E. and Laumond, C. (2000). Pathogenicity caused by high virulent and low virulent strains of *Steinernema carpocapsae* to *Galleria mellonella*. *J. Invertebr. Pathol.* **75**, 47-54.

Singh, N.K., Pakkianathan, B.C., Kumar, M., Prasad, T., Kannan, M., Konig, S. and Krishnan, M. (2013). Vitellogenin from the silkworm, *Bombyx mori*: an effective anti-bacterial agent. *PLoS One* **8**, e73005.

Sinpoo, C., Paxton, R.J., Disayathanoowat, T., Krongdang, S. and Chantawannakul, P. (2018). Impact of *Nosema ceranae* and *Nosema apis* on individual worker bees of the two host species (*Apis cerana* and *Apis mellifera*) and regulation of host immune response. *J. Insect Physiol.* **105**, 1-8.

Socha, R. and Kodrík, D. (1999). Differences in adipokinetic response of *Pyrrhocoris apterus* (Heteroptera) in relation to wing dimorphism and diapause. *Physiol. Entomol.* **24**, 278-284.

Socha, R., Šula, J., Kodrík, D. and Gelbič, I. (1991). Hormonal control of vitellogenin synthesis in *Pyrrhocoris apterus*. *J. Insect Physiol.* **37**, 805-816.

Stoscheck, C.M. (1990). Quantitation of proteins. In *Methods in Enzymology* (ed. M.P. Deutscher), pp. 50-68. London, UK: Academic Press.

Towbin, H., Staehelin, T. and Gordon, J. (1979). Electrophoretic transfer of protein from polyacrylamide gel to nitrocellulose. *Proc. Natl. Acad. Sci. USA* **76**, 4350-4354.

Villar, G. and Grozinger, C.M. (2017). Primer effects of the honeybee, *Apis mellifera*, queen pheromone 9-ODA on drones. *Anim. Behav.* **127**, 271-279.

Wang, Z., Wilhelmsson, C., Hyršl, P., Loof, T.G., Dobeš, P., Klupp, M., Loseva, O., Mörgelin, M., Iklé, J., Cripps, R.M. et al. (2010). Pathogen entrapment by transglutaminase-A conserved early innate immune mechanism. *PLoS. Pathog.* **6**, 1-9.

Waterfield, N.R., Ciche, T. and Clarke, D. (2009). *Photorhabdus* and a host of hosts. *Annu. Rev. Microbiol.* **63**, 557-74.

Zhang, W.N., Xiao, H.J., Liang, G.M., Guo, Y.Y. and Wu, K.M. (2014). Tradeoff between reproduction and resistance evolution to Bt-toxin in *Helicoverpa armigera*: Regulated by vitellogenin gene expression. *Bull. Entomol. Res.* **104**, 444–452.

Zhang, W.N., Ma, L., Zhong, F., Wang, Y.N., Guo, Y.Y., Lu, Y.H. and Liang, G.M. (2015). Fitness costs of reproductive capacity and ovarian development in a Bt resistant strain of the cotton bollworm, *Helicoverpa armigera* (Hubner) (Lepidoptera: Noctuidae). Pest Manage. Sci. 71, 870–877.

Zimmermann, G. (2008). The entomopathogenic fungi *Isaria farinosa* (formerly *Paecilomyces farinosus*) and the *Isaria fumosorosea* species complex (formerly *Paecilomyces fumosoroseus*): biology, ecology and use in biological control. *Biocontrol Sci. Technol.* **18**, 865-901.

Figure legends:

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

Fig. 2. (A) SDS-PAGE of hemolymph proteins of *P. apterus*: St - MW standards; 1 - control 4-day old females (served for the vitellogenin identification); 2 - 8-day old males 1 day after the control treatment; 3 - 8-day old males 1 day after the *S. carpocapsae* treatment. Vitellogenin (Vg) bands are indicated by the arrow. (B) Western blotting of hemolymph proteins of *P. apterus*. For legends (1-3) see (A). Bands positively reacted with anti-Vg-antibody are indicated by the arrows; estimated MW: a - 180 kDa, b - 65 kDa and C - 24 kDa. (C) Relative level of vitellogenin bands (quantified from corresponding gels that are not shown) in hemolymph of 8-day old *P. apterus* males 1 day after the *S. carpocapsae* (EPN) or control treatments. Statistically significant difference between infected and control

groups at 1% level evaluated by Student's t-test is indicated by ** (n=4). The number above the bar represents fold-difference of the relative vitellogenin quantities between EPN group and control.

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

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

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

per each) and 1% level (B, n=4-5) evaluated by Student's t-test are indicated by * and **, respectively. The numbers above the bars represent fold-difference between the relevant groups.

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

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

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

Fig. 9. (A) SDS-PAGE of hemolymph proteins of *P. apterus* females: St - MW standards; 1 + 2 - control and *I. fumosorosea*- infected 2-day old females, 1-day

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

Fig. 10. The effect of *I. fumosorosea* on mortality of *P. apterus* males (7-day old treated) and females (4-day old treated) 1-3-day after the infection; mortality in controls is not shown being null or negligible. No statistically significant difference between males and females evaluated by two-way ANOVA at 5% were recorded (n=10-15).

Fig. 1

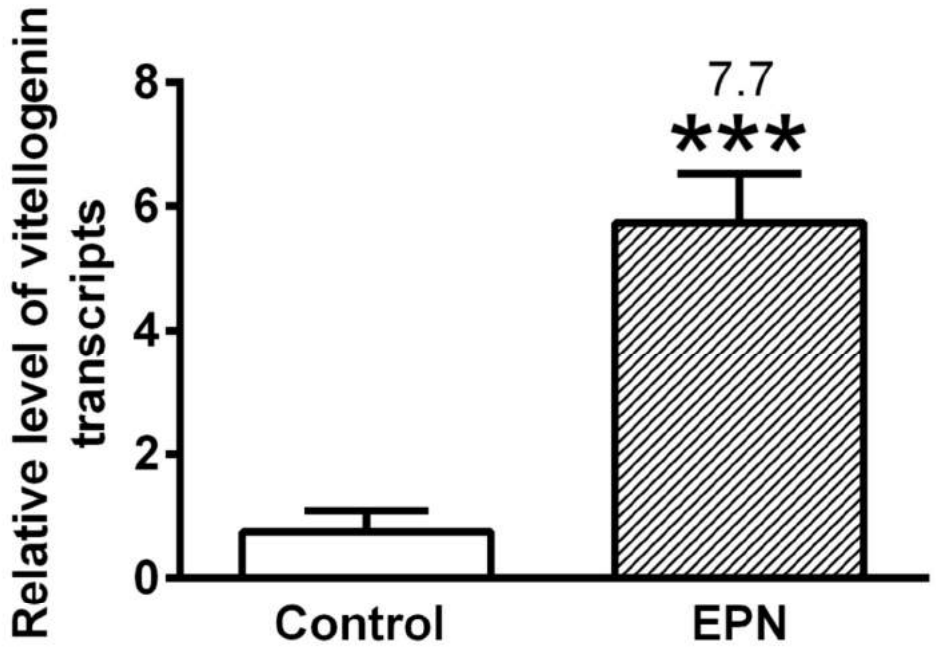

Relative level of vitellogenin transcripts
8
6
4
2
0
7.7
***
Control
EPN

Fig. 2

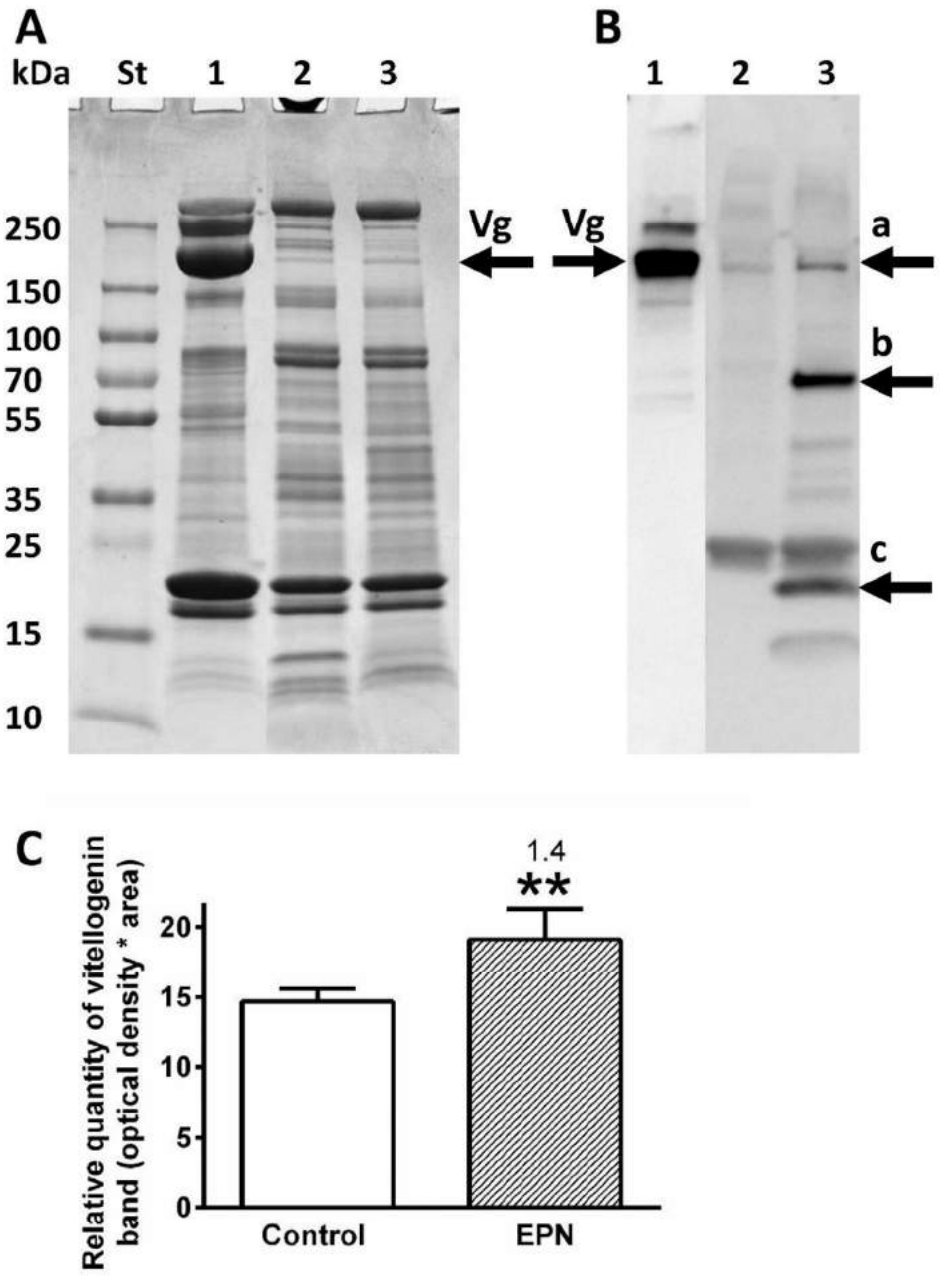

A
kDa
St
1
2
3
250
150
100
70
55
35
25
15
10
Vg
Vg
B
1
2
3
a
b
c
C
Relative quantity of vitellogenin band (optical density * area)
20
15
10
5
0
1.4
**
Control
EPN

Fig. 3

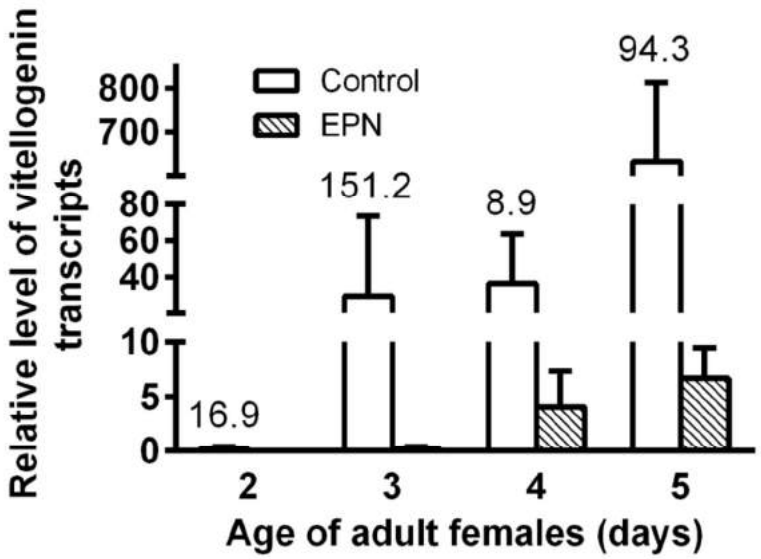

Relative level of vitellogenin transcripts
800
700
80
60
40
10
5
0
Control
EPN
16.9
151.2
8.9
94.3
2
3
4
5
Age of adult females (days)

Fig. 4

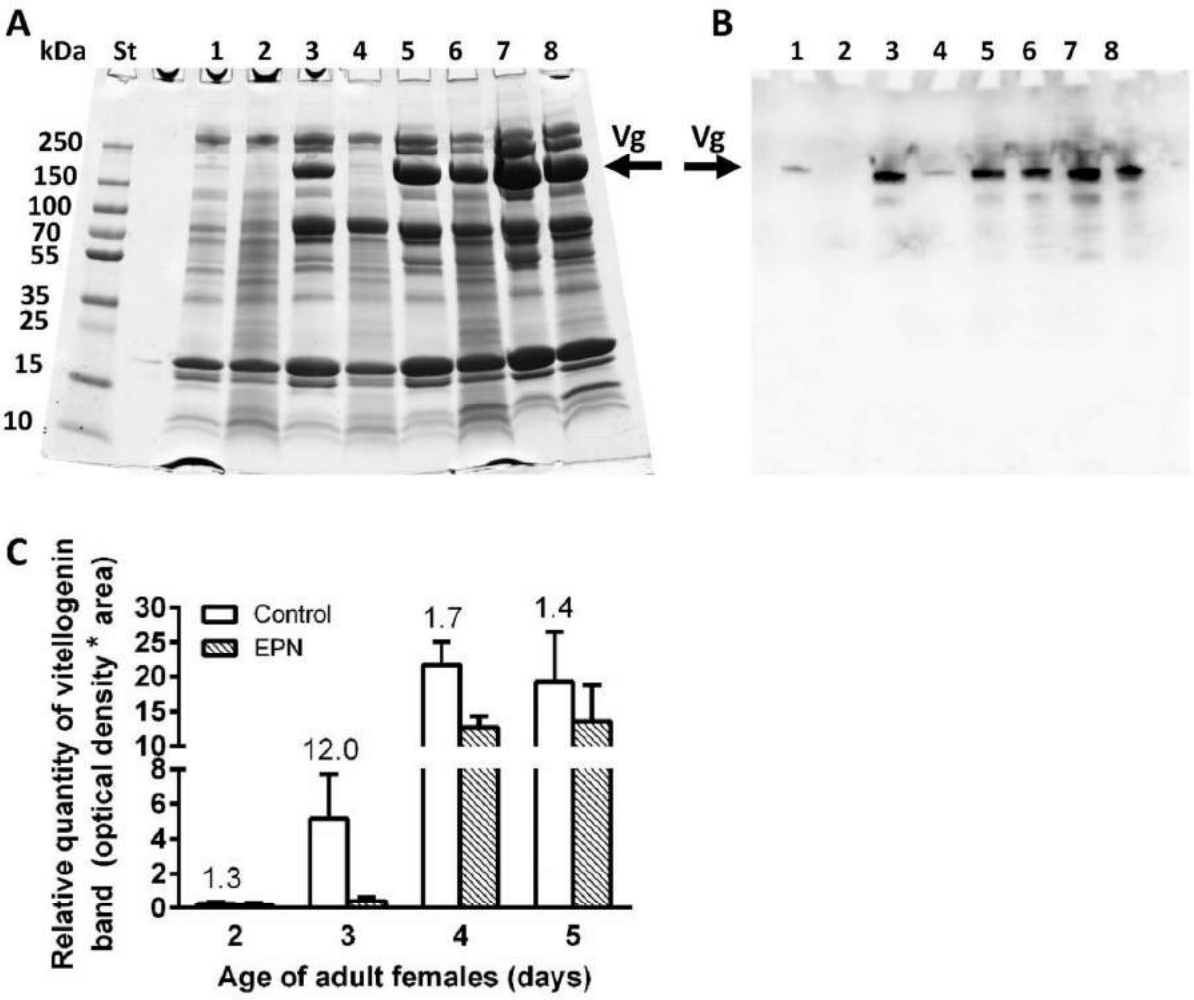

A
kDa
St
1 2 3 4 5 6 7 8
250
150
100
70
55
35
25
15
10
Vg
Vg
B
1 2 3 4 5 6 7 8
C
Relative quantity of vitellogenin band (optical density * area)
Control
EPN
1.3
12.0
1.7
1.4
Age of adult females (days)

Fig. 5

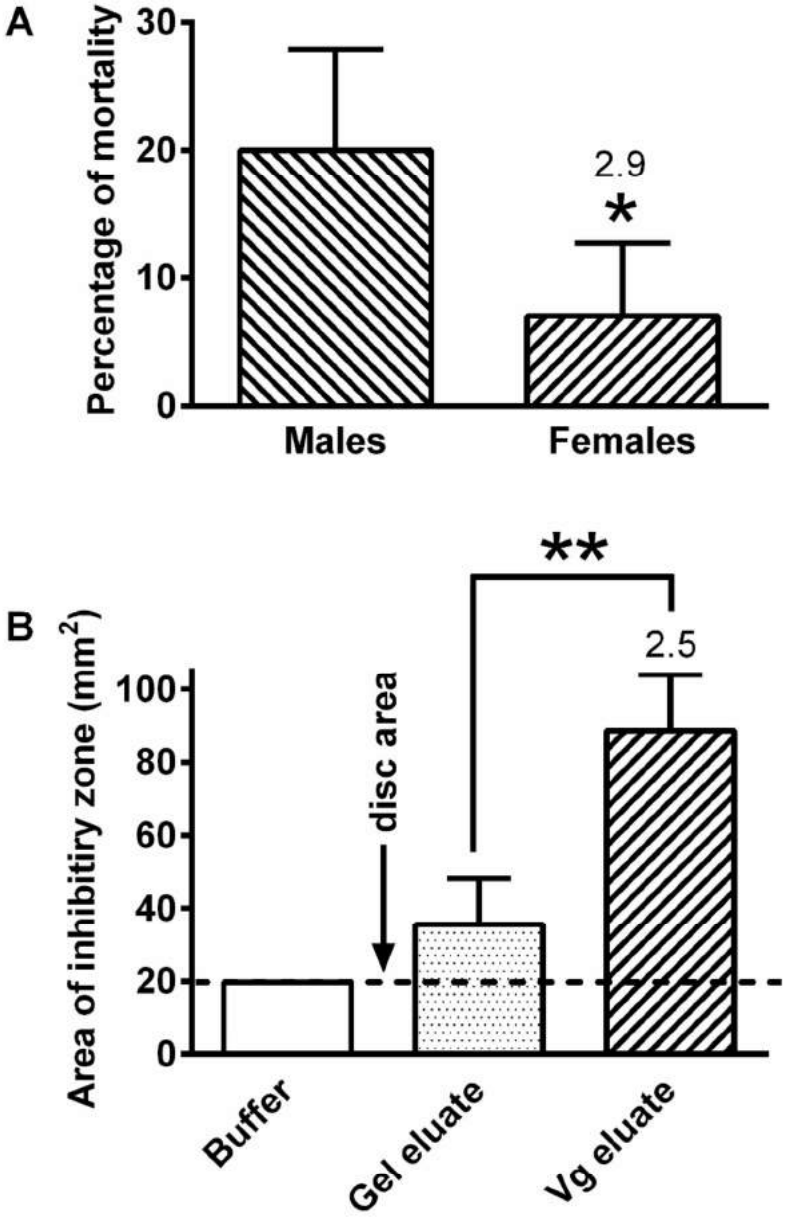

A
Percentage of mortality
30
20
10
0
2.9
*
Males
Females
B
**
2.5
Area of inhibitiry zone (mm2)
100
80
60
40
20
0
disc area
Buffer
Gel eluate
Vg eluate

Fig. 6

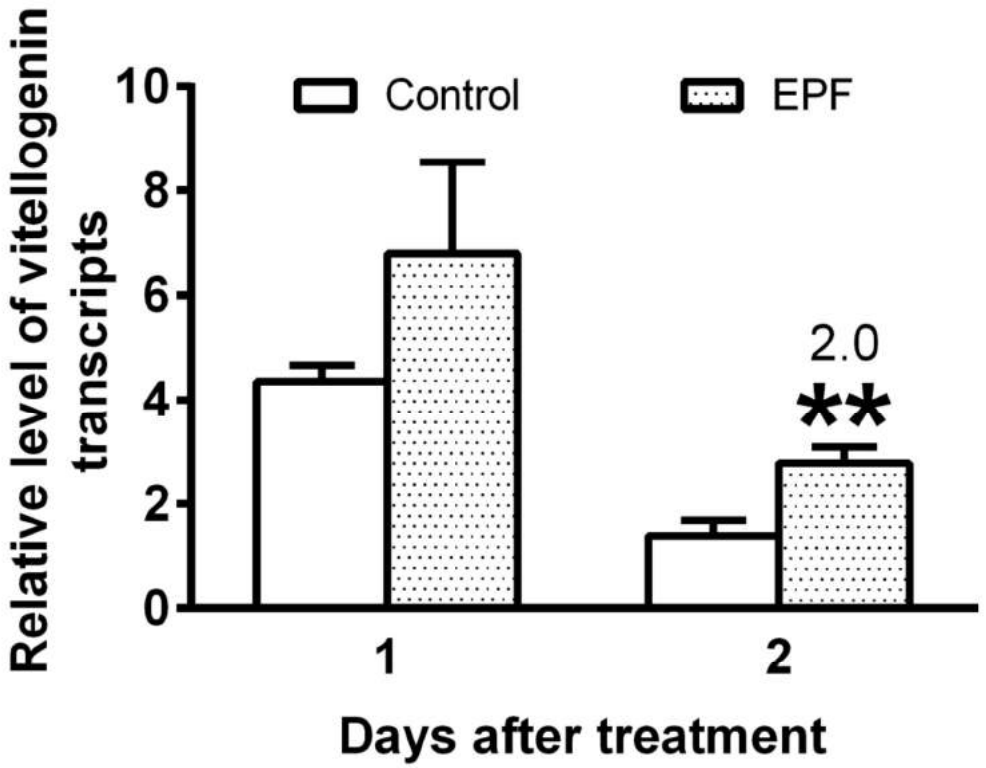

Relative level of vitellogenin transcripts
10
8
6
4
2
0
Control
EPF
2.0
**
1
2
Days after treatment

Fig. 7

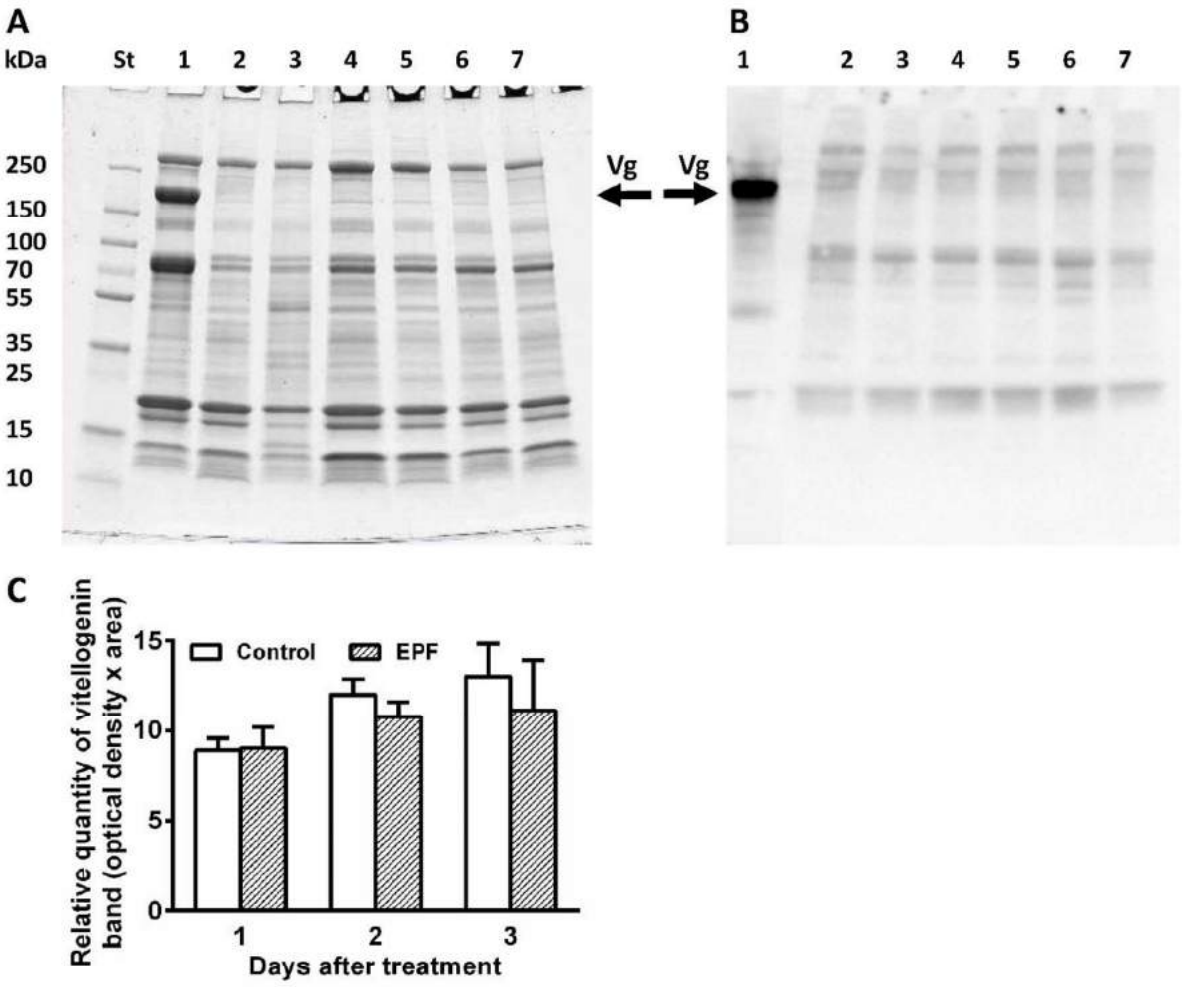

A
kDa
St
1
2
3
4
5
6
7
250
150
100
70
55
35
25
15
10
Vg
Vg
B
1
2
3
4
5
6
7
C
Relative quantity of vitellogenin band (optical density x area)
Control
EPF
15
10
5
0
1
2
3
Days after treatment

Fig. 8

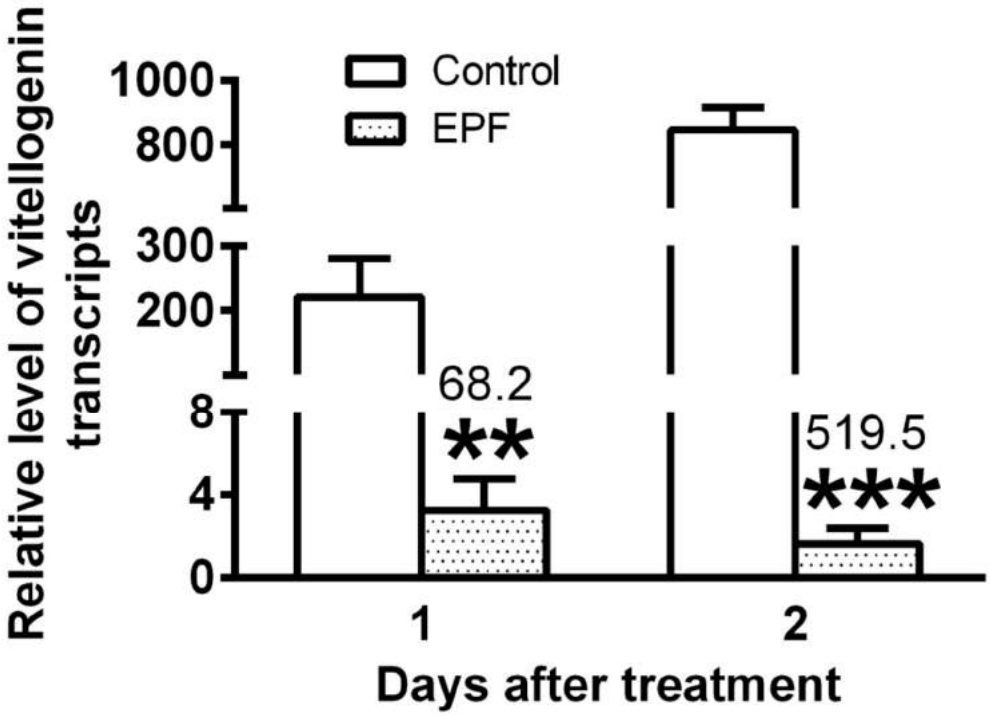

Relative level of vitellogenin transcripts
1000
800
300
200
8
4
0
Control
EPF
68.2
**
519.5
***
1
2
Days after treatment

Fig. 9

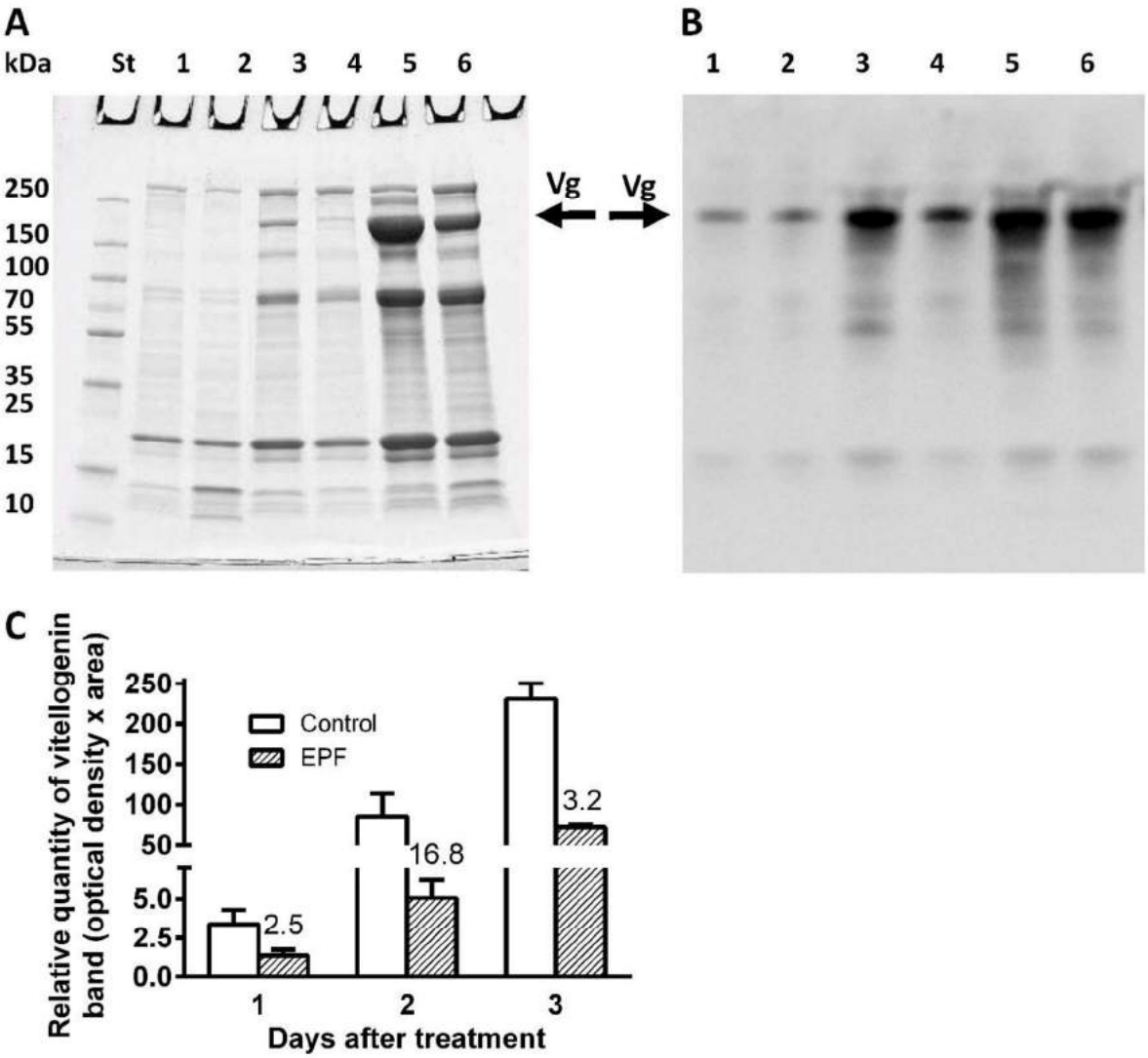
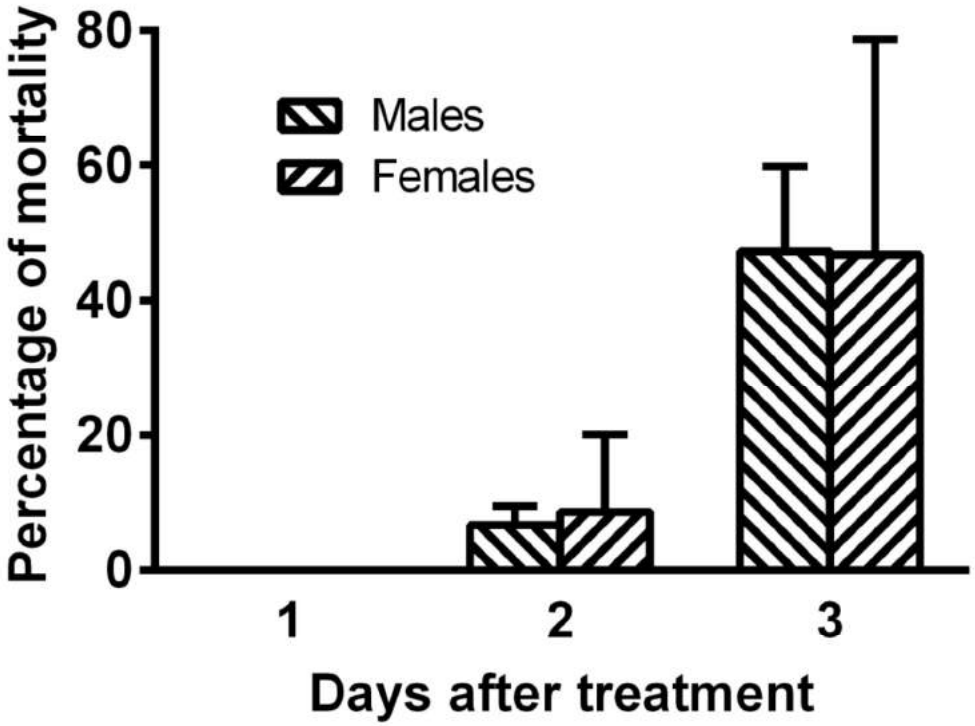

A
kDa
St
1
2
3
4
5
6
250
150
100
70
55
35
25
15
10
Vg
Vg
B
1
2
3
4
5
6
C
Relative quantity of vitellogenin band (optical density x area)
250
200
150
100
50
5.0
2.5
0.0
Control
EPF
2.5
16.8
3.2
1
2
3
Days after treatment

Fig. 10


Percentage of mortality
80
60
40
20
0
Males
Females
1
2
3
Days after treatment

6. Appendix

6.1. List of abbreviations

AdoR – Adenosine receptor

$AdoR^{1}$– Mutation in AdoR gene

ADP – Adenosine diphosphate

AHK $^{1}AdoR^{1}$– Mutation in AKH & AdoR genes

AICAR – 5-aminoimidazole-4 carboxamide ribonucleotide

AKH – Adipokinetic hormone

AKH^{1}– Mutation in AKH gene

AKHR – AKH receptor

AMP – Adenosine monophosphate

AMP – Antimicrobial peptide

AST – Allatostatin

AT – Allatotropin

ATP – Adenosine triphosphate

CA – Corpora allata

cAMP – Cyclic adenosine monophosphate

CAT – Catalase

CC – Corpora cardiaca

CNS – Central nervous system

Cry 3Aa – Cry 3Aa-Bacillus thuringiensis toxin

DAG – Diacylglycerol

DOPA – Dihydroxyphenyl alanine

Ec – Ecdysteroids;

Ecto-5′NTase – Ecto-5′-nucleotidase

Ent1 – Extracellular nucleoside transporter

EPN– Entomopathogenic nematode

FAICAR – Formyl 5-aminoimidazole-4 carboxamide ribonucleotide

GDP – Guanosine diphosphate

GNA – *Galanthus nivalis* agglutinin toxins

GPCR – G protein coupled receptor

GSH – Reduced glutathione

GST – Glutathione S-transferase

GTP – Guanosine triphosphate

G_{α} – Alpha subunit of G-protein

$G_{\beta\gamma}$ – Beta-gamma subunits of G-protein

HTH – Hypertrehalosemic hormone

IJ – Infective juvenile

ILPs – Insulin-like peptides

IMP – Inosine monophosphate

IP_3 – Inositol 1,4,5-trisphosphate

JH – Juvenile hormone

LPS – Lipopolysaccharide

OEH – Ovarian ecdysteroidogenic hormone

OS – Oxidative stress

PIP_2 – Phosphatidylinositol 4,5, bisphosphate

PKC– Protein kinase C

ProPO – Prophenoloxidase

RNS – Reactive nitrogen species

ROS – Reactive oxygen species

SOD – Superoxide dismutase

TMOF – Tripsin-modulating oostatic factor

Vg – Vitellogenin

w^{1118} – white control flies

6.2. Curriculum vitae

Personal details:

Name & surname:	Emad Ibrahim
Date of Birth:	March 1st, 1986
Citizenship/Nationality:	Egyptian

WORK EXPERIENCE:

2018 – Present	Research Assistant at the Department of Biochemistry and Physiology, at the Laboratory of Insect Physiology, Institute of Entomology, Biology Centre AS CR, České Budějovice, CR.
2013 – Present	Assistant lecturer at the Department of Economic Entomology and Pesticides, Faculty of Agriculture, University of Cairo, Egypt.
2008 – 2013	Teaching Assistant at the Department of Economic Entomology and Pesticides, Faculty of Agriculture, University of Cairo, Egypt.

EDUCATION AND TRAINING:

2015 – Present	PhD student at University of South Bohemia, Faculty of Sciences, České Budějovice, CR & Department of Biochemistry and Physiology, at the Laboratory of Insect Physiology, Institute of Entomology, Biology Centre AS CR.

Rerum naturalium doctor (RNDr.) 2018
Faculty of Sciences, University of South Bohemia, České Budějovice, CR & Department of Biochemistry and Physiology, at the Laboratory of Insect Physiology, Institute of Entomology, Biology Centre AS CR.

September 30th, 2017 - November 26th,2017
Short scientific stay at the Department of Molecular Biosciences, Stockholm University.

2011 – 2013	Graduated MSc. in Plant Protection Department, Faculty of Agrobiology, Food and Natural Resources, Czech University of Life Sciences, Czech Republic.
2010 – 2011	Czech language training at Institute for Language and Preparatory Studies, Study Centre Mariánské Lázně, Charles University in Prague, Czech Republic.

2003 – 2007 Graduated BSc. in Plant Protection Department„ Faculty of Agriculture, University of Cairo, Egypt.

TEACHING EXPERIENCE:

(2008 – 2010) & (2013 – 2015)
Teaching assistant in practical course: Principles of plant protection and Insect Physiology (Department of Economic Entomology and Pesticides, Faculty of Agriculture, University of Cairo, Egypt).

ADDITIONAL INFORMATION:

Publications

Ibrahim E., Dobeš P., Kunc M, Hyršl P., Kodrík D. (2018) Adipokinetic hormone and adenosine interfere with nematobacterial infection and locomotion in *Drosophila melanogaster*. *J. Insect Physiol.*107: 167-174.

Ibrahim E., Hejníková M., Shaik H. A., Doležel D., Kodrík D. (2017) Adipokinetic hormone activities in insect body infected by entomopathogenic nematode. *J. Insect Physiol.* 98: 347-355.

Salem M.S., Belal M.H., Nour M.E., **Ibrahim E.** (2012) Detection of a chemical marker from ovipositing females in *Rhynchophorus ferrugineus* Olivier (Coleoptera, Curculionidae). *Adv. Environ. Biol.* 6: 2164-2169.

Submitted manuscript

Kodrík D., **Ibrahim E**., Gautam U. K., Frydrychová R., Bednářová A., Krištůfek V., and Jedlička P. Role of vitellogenin in nematodal and fungal infections in insects. *J. Exp. Biol.*

Conferences

Hyršl P., **Ibrahim E.,** Dobeš P., Kunz M., Kodrík D. (2017) Adipokinetic hormone and adenosine in *Drosophila melanogaster* as a part of defence reaction against nematobacterial infection. *25th European Drosophila Research Conference*, London, United Kingdom, September 22 - 25, Abstract No. P280, p. 268.

Gautam U. K., **Ibrahim E.,** Zemanová M., Hejníková M., Žurovec M., Kodrík D. (2017) Insect model species and their mutants used for adipokinetic hormone studies. 93. *Fyziologické dny - Konference české a slovenské fyziologické společnosti, Košice, Slovensko,* 31. ledna - 2. února, Švorc P. a

Kundrík M. (editoři), ISBN 978-80-89046-98-0, str. 41.

Ibrahim E., Dobeš P., Hyršl P., Kodrík D. (2016) Defence reaction against nematobacterial infection in *Drosophila melanogaster*: a role for the adipokinetic hormone and adenosine. *28th Conference of European Comparative Endocrinologists*, Leuven, Belgium, August 21 - 25, Abstract No. PO 77, p. 324.

e-mailová adresa: omad_eng86@agr.cu.edu.eg

Study of adipokinetic hormone role in insects stressed by entomopathogenic nematodes

responses in insects. Ph.D. Thesis, 2019

Printed in České Budějovice, CR.

University of South Bohemia in České Budějovice

Faculty of Science, Branišovská 1760

CZ-37005 České Budějovice, Czech Republic

Phone: +420 387 776 201

www.prf.jcu.cz, e-mail: sekret-fpr@prf.jcu.cz