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Adipokinetic hormone activities in insect body infected by entomopathogenic nematode

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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,

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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-pro-hormones (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.

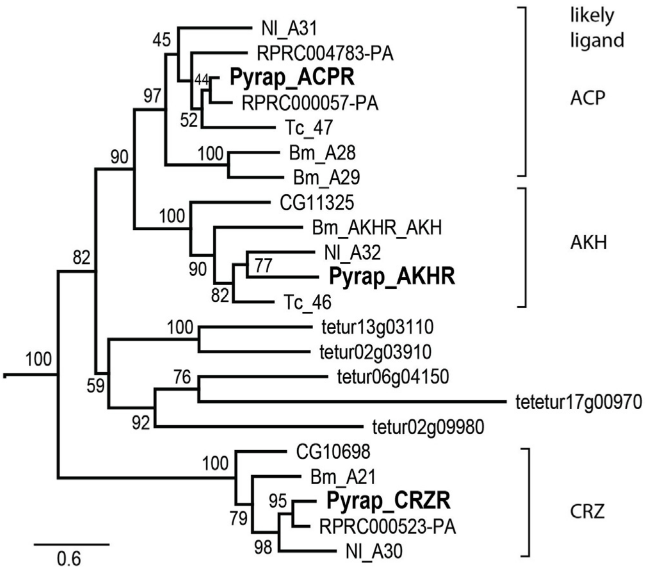

Fig. 1. *P. apterus* contains clear homologs of adipokinetic hormone receptor (Pyrap-AKHR), corazonin receptor (Pyrap-CRZR) and AKH/corazonin-related peptide receptor (Pyrap-ACPR). Phylogenetic tree was obtained from RAxML 7.2.8 analysis of protein sequences under LG + Gamma substitutional model. Bootstrap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

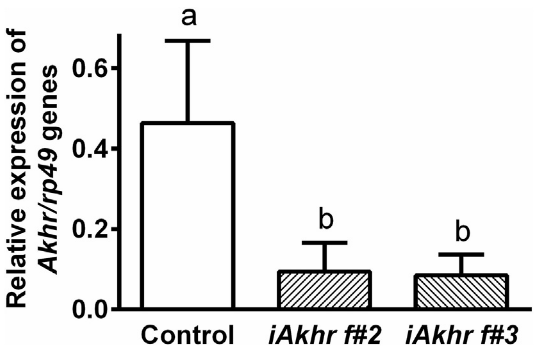

Fig. 2. RNAi mediated knockdown of *Akhr f#2* and *Akhr f#3* gene expression. All data were normalised using expression of *rp49* gene. Statistically significant differences among the groups at the 5% level evaluated by one-way ANOVA with the Tukey's post-test are indicated by different letters; bars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

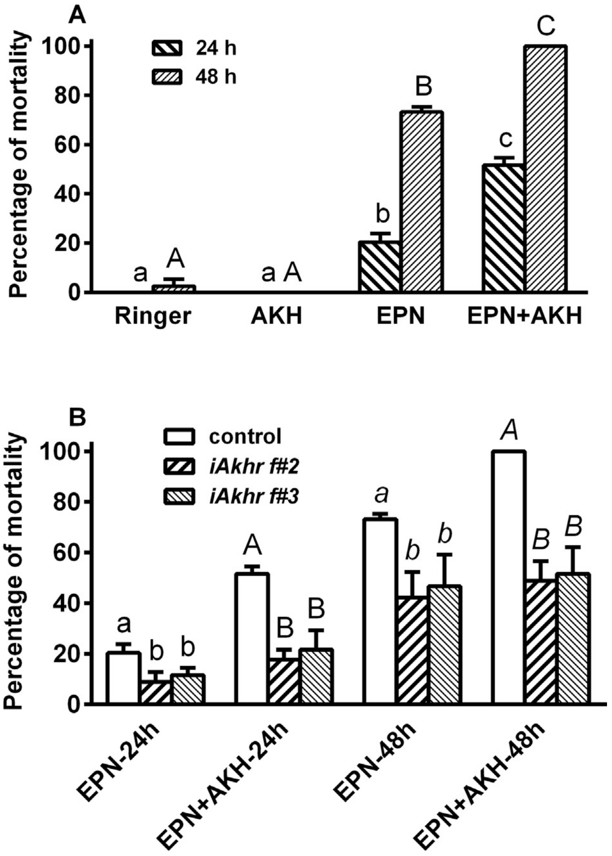

Fig. 3. (A) The effect of entomopathogenic nematode *S. carpocapsae* (EPN; 100 ind./insect) and Pyrap-AKH treatment (AKH; 10 pmol) on mortality of *P. apterus* adults (with normal *Akhr* expression) 24 or 48 h after the treatment. (B) The same effect on *P. apterus* adults with reduced *Akhr* expression (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.

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}.

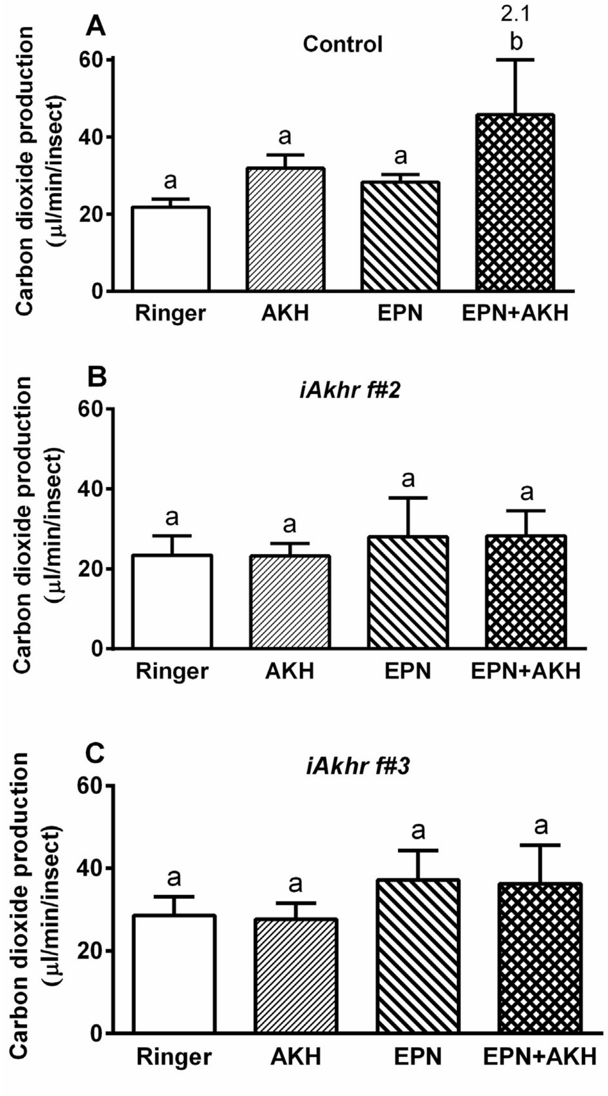

Fig. 4. (A) The effect of entomopathogenic nematode *S. carpocapsae* (EPN; 100 ind./insect) and Pyrap-AKH treatment (AKH; 10 pmol) on carbon dioxide production in *P. apterus* (control) adults 24 h after the nematode and/or 90 min after the Pyrap-AKH treatments. The number above the column represents 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.

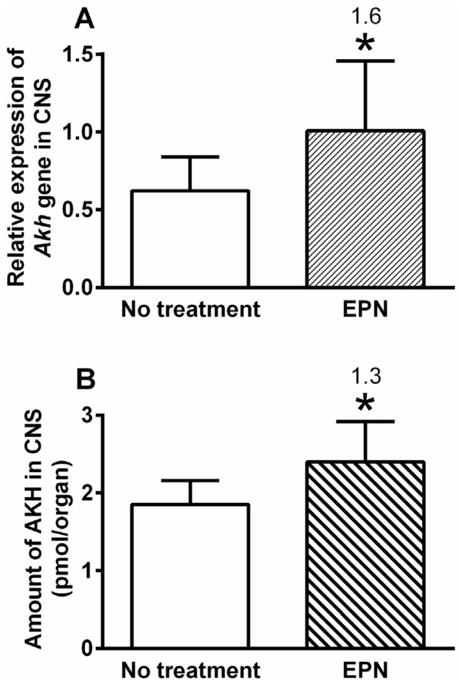

Fig. 5. The effect of entomopathogenic nematode *S. carpocapsae* (EPN; 100 ind./insect) treatment on (A) *Pyrap-Akh* gene expression, and (B) on total AKH amount in *P. apterus* adult CNS 24 h after the treatment. Statistically significant differences between the experimental groups and untreated controls 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.

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.

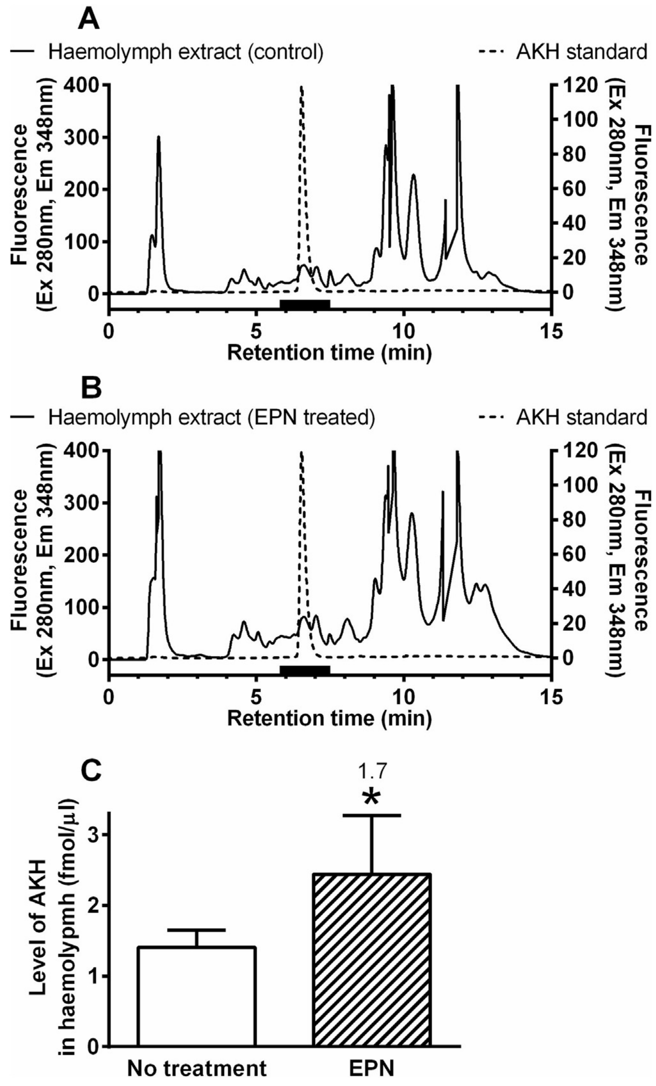

Fig. 6. The RP HPLC elution profiles of an prepurified extract of (A) 125 µl haemolymph from (untreated) *P. apterus* adults, and (B) 175 µl of haemolymph from adults 24 h after the treatment by *S. carpocapsae* (EPN; 100 ind./insect) (solid lines), and profiles of a mixture of AKH standards (Pyrap-AKH – 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.

2.6. 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

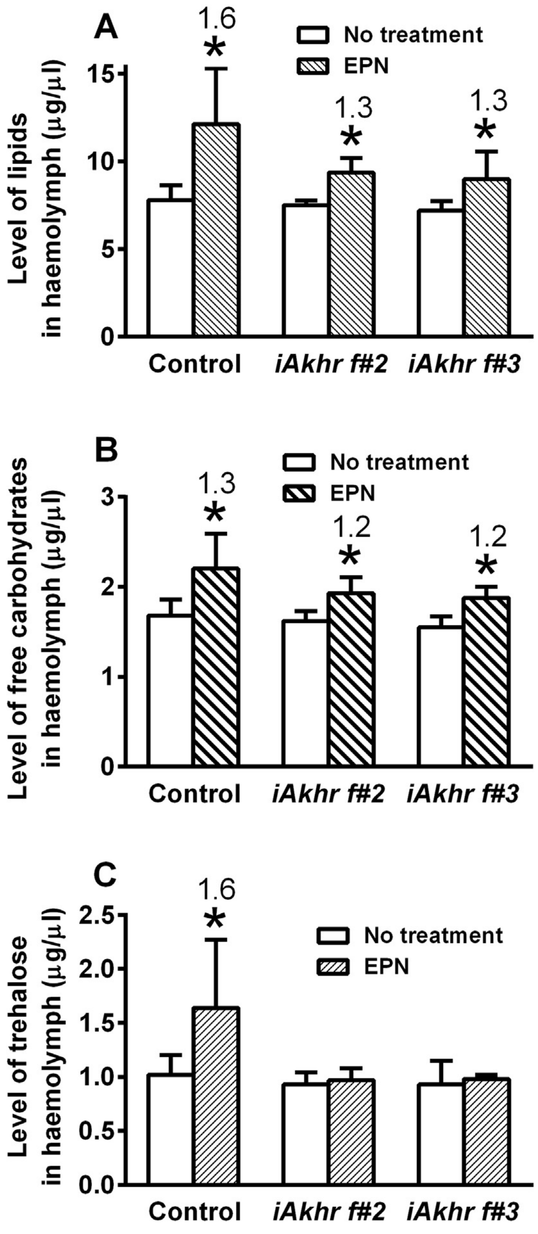

Fig. 7. The effect of entomopathogenic nematode *S. carpocapsae* (EPN; 100 ind./insect) treatment on lipid (A), free carbohydrate (B) and trehalose (C) levels in *P. apterus* adult haemolymph 24 h after the treatment. Both control firebugs and those with reduced *Akhr* expression were used for the experiments. 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.

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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.

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