University of South Bohemia in České Budějovice

Faculty of Science

Role of adipokinetic hormone in the Colorado potato beetle, *Leptinotarsa decemlineata* infected with the entomopathogenic nematode *Steinernema carpocapsae*.

RNDr. Thesis

Mgr. Daniela Hlávková

České Budějovice 2023

Hlávková, D. 2022 Role of adipokinetic hormone in the Colorado potato beetle, *Leptinotarsa decemlineata* infected with the entomopathogenic nematode *Steinernema carpocapsae*. RNDr. Thesis, in English, University of South Bohemia, Faculty of Science, České Budějovice, Czech Republic, 12pp.

Annotation

The study investigates the involvement of adipokinetic hormone (AKH) in the Colorado potato beetle's *Leptinotarsa decemlineata* (Say, 1824) (Coleoptera: Chrysomelidae) responsive reactions to infection of the entomopathogenic nematode *Steinernema carpocapsae* (Weiser, 1955) (Rhabditida: Steinernematidae). The findings show that nematode presence doubles the amount of AKH in the central nervous system of *L. decemlineata*, indicating mobilization of anti-stress reactions in the body. Moreover, the external co-application of Peram-CAH-II with the nematode significantly increased beetle mortality. Therefore, the paper investigates possible mechanisms underlying these phenomena and suggests that the nematodes may benefit from the observed mobilization of metabolites from the fat body into the Peram-CAH-II-induced hemolymph, which provides them with a more nutrient-dense substrate for propagation. These findings could serve in the development suitable insect pest control methods in the future.

Prohlášení: Prohlašuji, že jsem autorem této kvalifikační práce a že jsem ji vypracovala pouze s použitím pramenů a literatury uvedených v seznamu použitých zdrojů.

V Českých Budějovicích, dne 5. 9. 2023

Hlávková

.....

Daniela Hlávková

Tato práce je založena na následující publikaci:

Hlávková D., Skoková Habuštová O., Půža V., Vinokurov K., Kodrík D. (2022) Role of adipokinetic hormone in the Colorado potato beetle, *Leptinotarsa decemlineata* infected with the entomopathogenic nematode *Steinernema carpocapsae*. Comparative Biochemistry and Physiology C 262: 109466. DOI: https://doi.org/10.1016/j.cbpc.2022.109466 (IF= 4.52, 2023)

Prohlašuji, že jsem se významně podílela na všech činnostech souvisejících s publikováním předkládané práce. Připravovala a prováděla jsem experimenty, zpracovávala vzorky a prováděla analýzy, podílela se na statistickém vyhodnocení, přípravě manuskriptu. Můj celkový podíl na této publikaci byl 50 %.

.....

Daniela Hlávková

Souhlasím s výše uvedeným prohlášením.

Za všechny spoluautory

.....

prof. RNDr. Dalibor Kodrík, CSc.

Comparative Biochemistry and Physiology, Part C 262 (2022) 109466

Contents lists available at ScienceDirect

Comparative Biochemistry and Physiology, Part C

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

Role of adipokinetic hormone in the Colorado potato beetle, *Leptinotarsa decemlineata* infected with the entomopathogenic nematode *Steinernema carpocapsae*

Daniela Hlávková [a,b], Oxana Skoková Habuštová [a], Vladimír Půža [a], Konstantin Vinokurov [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 31a, 370 05 České Budějovice, Czech Republic

ARTICLE INFO

Edited by Martin Grosell

Keywords:
AKH
Metabolism
Mortality
Nutrients
Vitellogenin

ABSTRACT

The effects of the entomopathogenic nematode *Steinernema carpocapsae* on the Colorado potato beetle (CPB) *Leptinotarsa decemlineata* and the involvement of adipokinetic hormone (AKH) in the responsive reactions were examined in this study. It was observed that nematode application doubled the amount of AKH (Peram-CAH-I and Peram-CAH-II) in the central nervous system of *L. decemlineata*, indicating mobilization of anti-stress reactions in the body. Furthermore, the external co-application of Peram-CAH-II with the nematode significantly increased beetle mortality (5.6 and 1.8 times, 1 and 2 days after application, respectively). The mechanism underlying this phenomenon was investigated. As the effect on gut characteristics was equivocal, it was assumed that the nematodes profited from the observed mobilization of metabolites from the fat body into the Peram-CAH-II-induced hemolymph. This phenomenon supplied nematodes with a more nutrient-dense substrate on which they propagated. Furthermore, Peram-CAH-II lowered vitellogenin expression in the fat body, particularly in males, thus limiting the anti-pathogen defense capacity of the protein. However, there could be other possible mechanisms underpinning this chain of events. The findings could be theoretically intriguing but could also aid in developing real insect pest control methods in the future.

1. Introduction

The Colorado potato beetle (CPB), *Leptinotarsa decemlineata* Say (Chrysomelidae), is a notable agricultural pest in numerous regions of the world (Jolivet, 1991). CPB larvae and adults feed on potatoes, eggplants, and a few other crops, and this species produces one to three generations per season. CPB overwinters as non-reproducing diapausing adults. Although there is marked amount of information on its development, reproduction, and other biological aspects, numerous other questions remain unresolved. Therefore, general information on CPB is welcome, especially that potentially important for developing novel strategies to control pest populations. In this study, we focused on monitoring several physiological functions in CPB infected with the entomopathogenic nematode *Steinernema carpocapsae*.

Entomopathogenic nematodes (EPNs) are multicellular parasites that form highly pathogenic nematobacterial complexes with specific symbiotic entomopathogenic bacteria species. These complexes release several toxins that kill infected insects during an EPN attack (Simões et al., 2000; Duchaud et al., 2003). Despite using strategies similar to related human pathogens (Castillo et al., 2011; Chaston et al., 2011), these complexes are not harmful to humans. They have recently become popular for the biological control of various insect pests (coleopterans, lepidopterans, and dipterans; Ehlers, 2001, 2003; Inman et al., 2012). Extensive research in the last few decades has suggested that EPNs could be effectively used in controlling CPB larvae and adults (e.g., Trdan et al., 2009; Hussein et al., 2016; Čačija et al., 2021; Půža et al., 2021).

Any entomopathogenic infection causes severe stress in insects. Insects must activate their defense reactions to eliminate or at least minimize the harmful impact of pathogens and their toxins on biochemical and physiological processes in their body. The defense response is controlled by nervous and hormonal defense systems; nevertheless, adipokinetic hormones (AKHs) play a crucial role in this process. In general, AKHs are pleiotropic neurohormones (neuropeptides) that maintain homeostasis in the insect body. They primarily

* 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).

https://doi.org/10.1016/j.cbpc.2022.109466
Received 8 June 2022; Received in revised form 24 August 2022; Accepted 8 September 2022
Available online 13 September 2022

trigger the mobilization of energy reserves (Gäde et al., 1997; Gäde and Goldsworthy, 2003), but also control several associated processes, such as immune response (Goldsworthy et al., 2002, 2005), neuronal signaling (Milde et al., 1995; Wicher et al., 2006), locomotion (Socha et al., 1999; Kodrík et al., 2000), food intake together with digestive processes in the insect gut (Kodrík et al., 2012; Bil et al., 2014, Bodláková et al., 2017), and anti-oxidative defense reactions (Kodrík, 2008; Kodrík et al., 2015a; Krishnan et al., 2007). Two different adipokinetic hormones, Peram-CAH-I (*Periplaneta americana* Cardioaccelerating Hormone-I: pGlu-Val-Thr-Phe-Thr-Pro-Asn-Trp-NH_2) and Peram-CAH-II (pGlu-Leu-Thr-Phe-Thr-Pro-Asn-Trp-NH_2), have been isolated from the corpora cardiaca of CPB (Gäde and Kellner, 1989).

Insect body intoxication by toxins, venoms, and pathogens usually increases *AKH* expression or peptide synthesis (Kodrík and Socha, 2005; Kodrík et al., 2015a, 2015b). Interestingly, the experimental co-application of toxins (Kodrík et al., 2015b) and pathogens (Ibrahim et al., 2017, 2018; Gautam et al., 2020a, 2020b) with synthetic AKH enhances the efficacy of both agents in the insect body. AKHs enhance the impact of toxins by stimulating metabolite exchange rates, especially in the early defense response stages (documented by enhancing carbon dioxide production) (Kodrík et al., 2015b), which may accelerate toxin penetration into tissues. The defensive reaction intensity is, therefore, quite intense and counterproductive, and consequently, kills the insects.

This study aimed to examine the effect of the nematode *S. carpocapsae* infection on selected metabolic activities of CPB to expand current knowledge about their interactions. The findings may be theoretically interesting but may also contribute to developing practical methods for insect pest management in the future.

2. Material and methods

2.1. Experimental insect

A stock culture of the Colorado potato beetle (CPB) *L. decemlineata* (Say) selected from wild populations collected from the organic farm (48.7091231N; 14.5788889E; elevation 675 m) in the vicinity of České Budějovice (South Bohemia, Czech Republic), was used in this study. Collected adults from the wild population were kept in a greenhouse at a constant temperature of 25 ± 1 °C and under long-day conditions (LD 16:8 h). They were fed on the leaves of the potato cultivar Magda. This cultivar was obtained from the Potato Research Institute, Havlíčkův Brod, Czech Republic, as a tiny plant, propagated as plant explants in agar cultures. Rooted explants were transferred to soil in the seedling plate and later replanted to 2 l containers.

2.2. Entomopathogenic nematodes

A total of 10 selected nematode species/strains from the collection of Laboratory of Entomopathogenic Nematodes (Institute of Entomology) were tested in a pilot (mortality) experiment to select the most suitable one for our study: *Steinernema feltiae* (KLEN, JAK, BĚL, 37 Ca and 626), *Steinernema carpocapsae* (MG 604, EGY 4 and 1343), *Steinernema arenarium* (SLOV) and *Heterorhabditis bacteriophora* (HB 221) (Table S1). For the following physiological experiments, just *S. carpocapsae* 1343 was used. Before the experiments, all nematodes were propagated using *Galleria mellonella* larvae, and 2–3 weeks old infective juveniles were used for the experiments. In the majority of experiments, the effect was determined 1-day after the treatment.

2.3. Nematode treatment and mortality determination

The suspension of *S. carpocapsae* juveniles was diluted in water and injected (through thoracic-abdominal intersegmental membrane) into bodies of both sexes of CPB adults in a volume 3 μl containing 25, 50, and 100 infective juveniles. The injection was employed because of the faster course of infection compared to natural infection (data not shown). Controls were injected by Ringer saline only. To determine mortality, 5–10 groups (each containing 25–33 insects) for each experimental group were monitored for 7-day post-treatment. A dose of 50 infective juveniles per CPB was chosen for physiological experiments (for reasons see Results).

2.4. Hormonal treatment

In some experiments, the effect of Peram-CAH-II injection (40 pmol; for reasons and other details see Kodrík et al., 2007) on infected CPBs was monitored. This peptide was prepared in the Polypeptide Laboratories, Praha, Czech Republic. To monitor the effect of both agents (*S. carpocapsae* and Peram-CAH-II), the tests were set up as follows: (a) control – Ringer saline, (b) Peram-CAH-II, (c) *S. carpocapsae*, and (d) *S. carpocapsae* + Peram-CAH-II together; the hormonal and nematodal injections were separated by 90 min to give the insects time to recover from the first injection. The effect was evaluated one day after treatment in all physiological experiments.

2.5. Extraction of AKHs and their quantification by ELISA

To extract the AKHs (Peram-CAH-I and -II together), the central nervous system (CNS) containing the brain with corpora cardiaca and corpora allata attached was dissected from the CPB head cut off from the rest of body under the Ringer saline. The AKHs were then extracted with 80 % methanol, the solution was evaporated in a vacuum centrifuge and the resulting pellet stored at −20 °C until needed.

The AKH content was determined using a competitive ELISA according to our protocol published earlier (Goldsworthy et al., 2002; Kodrík et al., 2007). The used antibody raised originally against Pyrap-AKH from the firebug *Pyrrhocoris apterus* (Kodrík et al., 2000, 2002) recognises well also both CPB AKHs (Peram-CAH-I and -II) (see Goldsworthy et al., 2002). In the ELISA test the equivalent of one CPB CNS per a well was used, with the first antibody diluted to 1:5000 (for more information see our earlier papers Goldsworthy et al. (2002) and Ibrahim et al. (2017)).

2.6. Metabolic rate measurement

The Li-7000 CO_2/H_2O analyser (Li-COR Biosciences, Lincoln, NE, USA) was used to measure the rate of carbon dioxide production by CPB individuals as described in our earlier paper (Kodrík et al., 2010). Seven individual CPB adults were measured separately in seven measuring chambers 1-day after the treatment for a period of 90 min, with 45 min serving as an accustoming phase and another 45 min as real measurement. Results were analysed by data acquisition software (Sable System, Las Vegas, Nevada, USA). The carbon dioxide production (VCO_2) was estimated from fractional carbon dioxide concentrations going in (FI) and coming out (FE) the chamber using the following equation (Withers, 1977) and expressed in μl min^{-1} mg $insect^{-1}$ units: $VCO_2 = (FECO_2 - FICO_2)$ f; where f is the flow rate in μl h^{-1}.

2.7. Activity of digestive enzymes in the midgut

The activities of glucosidases, lipases and proteases were determined in the CPB midgut. The organ was weighted, homogenized in appropriate buffer, centrifuged, and enzyme activity was determined.

- *Glucosidase assay* – the activity of α-glucosidase was performed with 4-methylumbelliferyl (MU) α-D-glucopyranoside as a substrate. The activity was expressed in pmol of 4-MU/min/mg of fresh gut (Kodrík et al., 2012).
- *Lipase assay* - the lipase activity was assessed with 4-methylumbelliferyl butyrate (4-MU butyrate) according to Roberts (1985) as modified by Kodrík et al. (2012). The activity was expressed in pmol of 4-MU/min/mg of fresh gut.

- *Protease assay* - the protease activity was assessed with the resorufin-casein kit (Roche) according to the manufacturer's instructions. The activity was expressed in units of proteolytic activity per mg of a fresh gut; this unit (U) was defined as the amount of enzyme (mg) which caused an increase in an optical density by 0.1 per min in 1 ml of the reaction mixture (Elpidina et al., 2001).

2.8. Spectrophotometric determination of nutrients

The levels of carbohydrates, glycogen, lipids, and proteins were determined in the CPB fat body and/or hemolymph 1-day after the nematode and/or hormonal treatments.

- *Fat body* – the tissue was homogenized in 70 % ethanol and the extract was used to determine carbohydrate (supernatant) and glycogen (sediment). Further, the lipids were extracted from the fat bodies by chloroform: methanol mixture, and proteins by 0.2 M tris HCl buffer, pH 7.8. For details of all tests see Mochanová et al. (2018).
- *Hemolymph* – the hemocytes were removed from hemolymph by centrifugation at 1000*g* for 2 min at 4 °C, and resulting serum was used for determination of the nutrients (carbohydrates, lipids, proteins).
- *Free carbohydrate determination* – the anthrone method (Carroll et al., 1956) as modified by Socha et al. (2004) was used for carbohydrate determination. The glucose standard curve was used for the final quantification.
- *Glycogen determination* – was done as described by Socha et al. (2004) employing the anthrone method for final quantification (see above).
- *Lipid determination* – was done by sulpho-phospho-vanillin method according to Zöllner and Kirsch (1962), as modified by Kodrík et al. (2000). The optical densities measured in a spectrophotometer were converted to lipids with the aid of a calibration curve based on known amounts of oleic acid.
- *Protein determination* – was done by the Bicinchoninic Acid Protein Assay Kit (Sigma-Aldrich) (Stoscheck, 1990). The bovine serum albumin standard curve was used for the quantification.

2.9. Quantification of vitellogenin gene expression

- *Tissue sample preparation* - the CPB fat bodies were dissected and separately placed into RNAase-free micro-centrifuge tubes (10 biological replicates from each group) with 200 µl of TRI Reagent® (Sigma-Aldrich Co., St. Louis, MO, the USA) and stored at −80 °C until further use.
- *RNA isolation and cDNA synthesis* - following the TRI Reagent® (Sigma-Aldrich) manufacturer's instructions, the fat bodies were homogenized, and the total RNA was extracted. After that, using the TURBO DNA-*free*™ Kit (Invitrogen, Thermo Fisher Scientific Baltics UAB, Vilnius, Lithuania) according to the manufacturer's protocol, the genomic DNA contamination in samples was eliminated. The cDNA templates were prepared using the RevertAid RT Reverse Transcription Kit (Thermo Fisher Scientific Baltics UAB, Vilnius, Lithuania) from 100 ng of previously obtained purified RNA samples using a random hexamer primers in Eppendorf MasterCycler Nexus (Eppendorf AG, Hamburg, Germany) according to the manufacturer's protocol. The vitellogenin (Vg) specific primers were designed based on *L. decemlineata* Vg sequences available in GenBank (GEEF01135093.1). The suitable reference gene (ribosomal protein 18, rp18) to normalize the expression data was chosen based on previous results (Shi et al., 2013). The effectivity of all primer pairs was verified before performing the q-RT-PCR. Selected primer sequences for performing q-RT-PCR are listed in the Table 1.
- *q-RT-PCR analysis* - the q-RT-PCR was performed using the CFX Connect™ Real-Time System (Bio-Rad Laboratories, Hercules, California, USA) with SYBR® Premix Ex Taq™ II (TIi RNaseH Plus) (TAKARA BIO INC., Shiga, Japan) and 40 nM of each primer. The 3-steps program was set: initial phase - 3 min at 95 °C; second step - 5 s at 95 °C, 30 s at 60 °C, 40 cycles. The final part - ramping from 65 °C to 95 °C by 0.5 °C every 5 s - served as a control to validate the amplification specificity. The primer effectivity was calculated from the constructed standard curve (5 serial dilutions). Then the generated Cq's values were used to obtain the relative expression of the targeted gene.

2.10. Data presentation and statistical analyses

The results were plotted using the graphic software Prism (GraphPad Software 6, San Diego, California). The points on the line graphs and the bar graphs represent the mean values ± SD. The number of repetitions is depicted in the figure legends. The statistical significance of the results was evaluated by (1) two-way ANOVA followed by Tukey's multiple comparison test (Fig. 1), (2) one-way ANOVA followed by Tukey's multiple comparison test (Figs. 2, 4–8), and (3) Student's *t*-test (Fig. 3).

3. Results

3.1. Effect of S. carpocapsae on CPB mortality

In the preliminary studies, we examined the influence of numerous

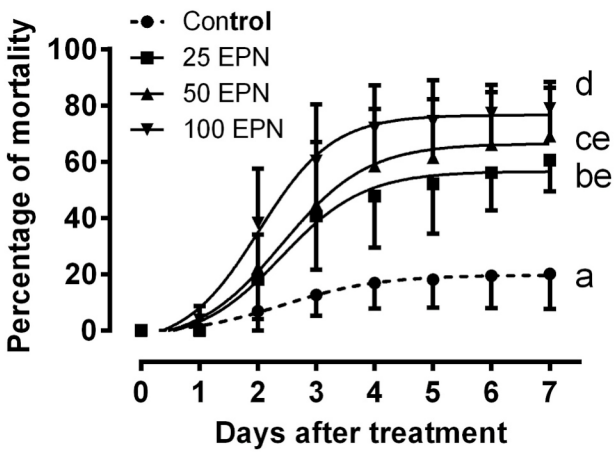

Fig. 1. The effect of injection of various doses of *S. carpocapsae* (EPN) on mortality of *L. decemlineata* adults 1–7-day after the treatment. Statistically significant differences among the doses (=the curves) at the 5 % level were evaluated using two-way ANOVA with Tukey's comparison test and are indicated by different letters (a, b, c, d, e); points on curves = mean ± SD, n = 5–6 groups with 25–33 individuals in each.

Table 1
Sequences of specific vitellogenin (Vg) and house-keeping genes, ribosomal protein 18 (rp18) primers (5′-3′ direction) used in q-RT-PCR.

Gene name		Primer sequence (5′-3′)	Amplicon size (bp)	Reference
Vg	Forward	AACCAGCAGAACGTGAACAG	157	
Vg	Reverse	ATCCTGTCAAAGTGCATGCC		
rp 18	Forward	ACTTCGTGTCACTGAAACTGC	252	Shi et al., 2013
rp 18	Reverse	TATCCGCACGACTTCCTGC		

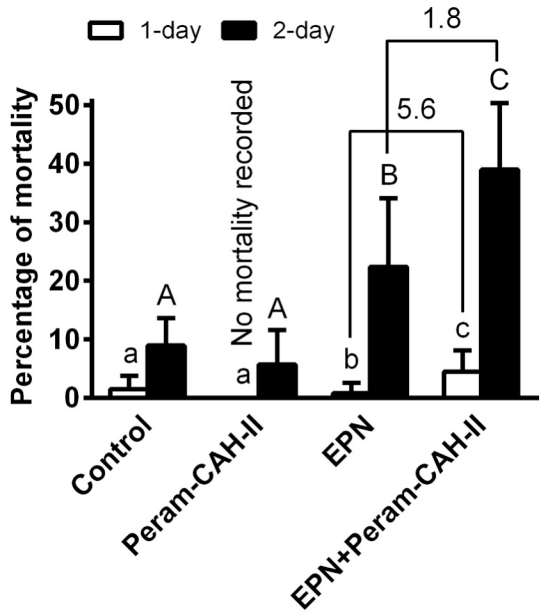

Fig. 2. The effect of injection of 50 individuals of *S. carpocapsae* (EPN) and 40 pmol Peram-CAH-II treatments on mortality of *L. decemlineata* 1st and 2nd day after the treatments. Statistically significant differences among the treatments at 5 % level evaluated by one-way ANOVA with the Tukey's post-test are indicated by different letters (a, b, c within 1-day; A,B,C within 2-day); bars = mean ± SD, n = 5–8 groups with 25–33 individuals in each. The numbers above the columns represent fold differences of mortality between the EPN and EPN + Peram-CAH-II treated groups.

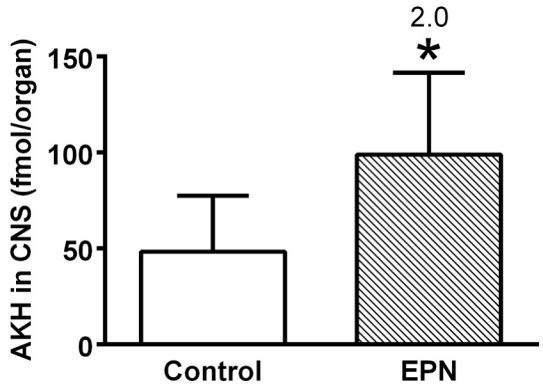

Fig. 3. The effect of injection of 50 individuals of *S. carpocapsae* (EPN) on AKH (Peram-CAH-I and –II) amount in *L. decemlineata* CNS 1st day after the treatment. Statistically significant difference between the groups at 5 % level evaluated by Student's t-test is indicated by *; bars = mean ± SD, n = 5–7. The number above the column represents the fold difference of AKH amount between the EPN and control groups.

entomopathogenic nematode species and strains on CPB mortality (Table S1) to select the best strain for our study. We chose *S. carpocapsae* strain 1343, owing to its consistent pathogenicity, for subsequent studies. According to the dosage response test, 50 nematodes per CPB individual were the most appropriate dose (Fig. 1). This dose was sufficiently high to induce stress (22.4 % mortality on the second day after infestation), but it left enough CPBs alive to conduct biochemical and physiological studies. Furthermore, co-application of 50 nematodes with 40 pmol Peram-CAH-II resulted in a significant increase in mortality, which was 5.6 and 1.8 times higher 1 and 2 days after application, respectively, than the mortality following the nematode treatment alone (Fig. 2). The injection of Ringer saline (control) or the hormone alone resulted in a less than 10 % mortality rate on both days studied.

3.2. *Effect of S. carpocapsae on AKH level in CNS*

As a result of *S. carpocapsae* treatment, infected insects are assumed to experience severe stress, followed by an increase in AKH synthesis. The AKH levels (Peram-CAH-I and -CAH-II) in the CNS were used to confirm this hypothesis. The results revealed that infected CPBs had a large, 2-fold increase in AKH levels (Fig. 3). Interestingly, there was no significant increase in AKH levels following the sham injection (intact insects vs. injury caused by the injection needle) (Fig. S1).

3.3. *Effect of S. carpocapsae on metabolic characteristics*

We intended to determine whether the application of *S. carpocapsae* and/or Peram-CAH-II affected overall metabolism. However, the results revealed that the differences in carbon dioxide production (as a marker of metabolic intensity) among our CPB experimental groups treated with the agents were minor and not significant (Fig. 4).

Furthermore, the impact of nematode and Peram-CAH-II on gut characteristics was not very dramatic (Fig. 5). The effect on gut mass was minimal and there were no significant changes among the groups (Fig. 5A). After treatment, digestive enzyme activity was disorganized. Glucosidase activity was curbed after hormonal treatment but not by nematodes (Fig. 5B), while the lipase activity significantly reduced after co-treatment with nematode and hormone (Fig. 5C), and protease activity was inhibited by Peram-CAH-II rather than by nematodes (Fig. 5D). Additionally, fat body mass did not change following nematode and hormonal treatments (Fig. 6A). However, the free carbohydrate levels increased following *S. carpocapsae* infection, but there was no stimulatory effect after co-treatment with *S. carpocapsae* and Peram-CAH-II, implying that the hormone counteracted the nematode-induced effects (Fig. 6B). Similarly, nematode treatment significantly increased fat body glycogen levels, whereas Peram-CAH-II had a minor and insignificant effect (Fig. 6C). Co-treatment only affected or reduced the level of fat body lipids (Fig. 6D), but not the level of proteins (Fig. 6E).

The levels of nutrients in the CPB hemolymph were changed similarly during the experimental treatment protocols. Nematodes significantly increased the level of free carbohydrates, as did Peram-CAH-II, to

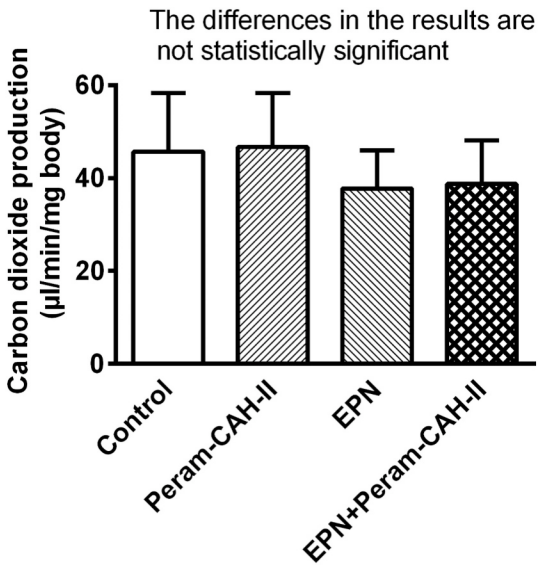

Fig. 4. The effect of injection of 50 individuals of *S. carpocapsae* (EPN) and 40 pmol Peram-CAH-II treatments on carbon dioxide production in *L. decemlineata* 1st day after the treatments. Statistical evaluation by one-way ANOVA with Tukey's post-test showed no significant differences among the results on 5 % level; bars = mean ± SD, n = 7.

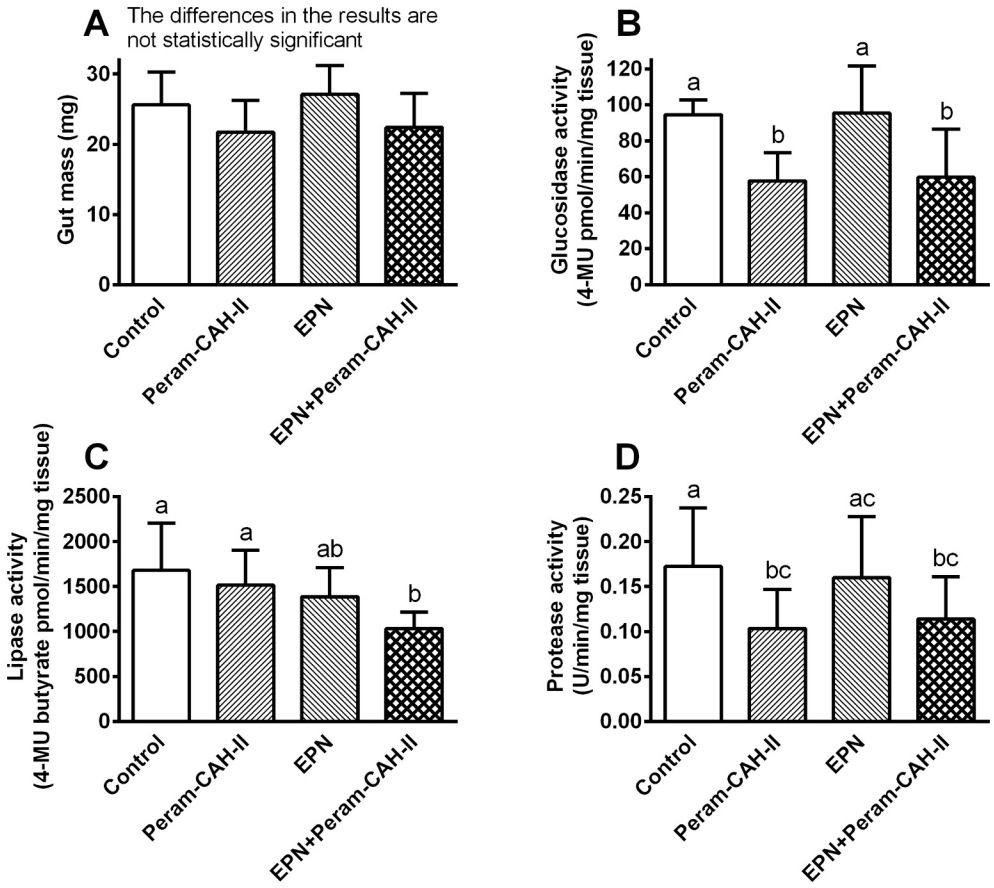

Fig. 5. The effect of injection of 50 individuals of *S. carpocapsae* (EPN) and 40 pmol Peram-CAH-II treatments on gut mass (A), and activity of gut α-glucosidases (B), lipases (C), and proteases (D) of *L. decemlineata* 1st day after the treatments. Statistically significant differences among the treatments at 5 % level evaluated by one-way ANOVA with Tukey's post-test are indicated by different letters (a, b, c); bars = mean ± SD, n = 10–15.

a lesser extent while still being significant. In the Peram-CAH-II + nematode co-treatment, the hormone partially eliminated the nematode-induced effect while maintaining the carbohydrate level at the same level as that after the hormonal treatment (Fig. 7A). Lipid levels significantly increased after *S. carpocapsae* infection, and it appears that AKH presence did not affect lipid levels in the hemolymph of both infected and uninfected CPBs (Fig. 7B). Only nematode infection significantly increased the hemolymph protein level, whereas the co-treatment of nematodes with Peram-CAH-II decreased the protein level to the same level as the control (Fig. 7C).

3.4. Effect of S. carpocapsae on vitellogenin expression

Using *S. carpocapsae* and/or Peram-CAH-II influenced vitellogenin (*Vg*) expression in both male and female CPB fat bodies. The *Vg* transcript increased about 3-fold in the male fat body after nematode treatment when compared to the level in control (Fig. 8A), but hormonal co-treatment stabilized the level back to the control level. Naturally, control females had a higher *Vg* transcription rate than control males (Fig. 8B); however, nematode infection resulted in an over 5-fold decrease; however, the difference due to high variability in controls was not significant. Hormonal treatment had no significant effects.

4. Discussion

4.1. Mortality and its hormonal enhancement

The effect of injecting different entomopathogenic nematode strains from the genera *Steinernema* and *Heterorhabditis* into CPB was examined in the pilot experiments of this study, and the mortality test revealed that *S. carpocapsae* 1343 was the most pathogenic. This strain was originally isolated in northern Bohemia, Czech Republic in 2014. The efficacy of 1343 strain in the CBP was recently established in a study by Půža et al. (2021), where it was found to be one of the most lethal strains against CPB adults via Petri dish infection experiments, and it also proved to be efficient against pupating beetles in pot experiments. We used a dose of 50 nematodes per beetle in all physiological experiments. This dose was close to the LD_{20}, which left a sufficient number of living individuals for the experiments.

The invasion of entomopathogenic nematodes into the insect body elicits a strong immune response at the humoral and cellular levels. This includes encapsulation by hemocytes and melanization (Dunphy and Thurston, 1990; Wang et al., 1994). Among other insects, Colorado potato beetles are more resistant to entomopathogenic nematodes (Armer et al., 2004) and capable of effective encapsulation of penetrating nematodes (Thurston et al., 1994). Generally, insect defense reactions against pathogens and toxins are controlled by neural and hormonal (neurohormonal) systems led by AKHs. Infection and intoxication of the insect body are accompanied by AKH gene activation and an increase in AKH levels in the CNS (Fig. 3) and/or hemolymph. Numerous examples of this phenomenon have been reported in the literature. For instance, *S. carpocapsae* infection resulted in a large increase in Pyrap-AKH and Peram-CAH-II gene expression as well as increased levels of these adipokinetic peptides in the CNS and hemolymph of the firebug *Pyrrhocoris apterus* (Ibrahim et al., 2017). A similar effect was observed in the fruit fly *Drosophila melanogaster* (Ibrahim et al., 2018). In the firebug *P. apterus*, aphid *Acyrthosiphon pisum*, American cockroach *Periplaneta americana*, and larvae of lepidopteran

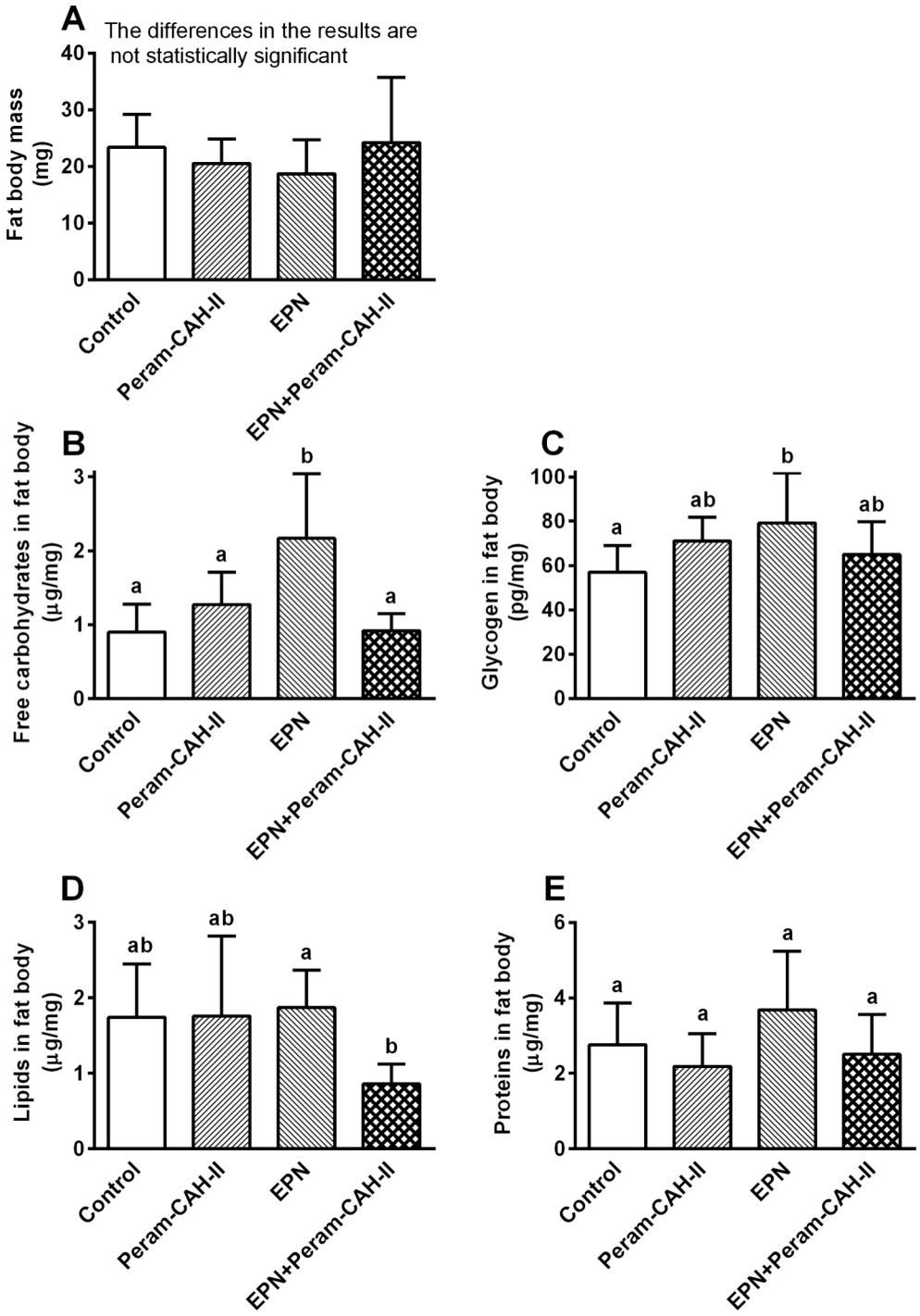

Fig. 6. The effect of injection of 50 individuals of *S. carpocapsae* (EPN) and 40 pmol Peram-CAH-II treatments on fat body mass (A), and level of fat body carbohydrates (B), glycogen (C), lipids (D), and proteins (E) of *L. decemlineata* 1st day after the treatments. Statistically significant differences among the treatments at 5 % level evaluated by one-way ANOVA with Tukey's post-test are indicated by different letters (a, b); bars = mean ± SD, n = 8–10.

species *Spodoptera littoralis*, an infection caused by the entomopathogenic fungus *Isaria fumosorosea* enhanced AKH characteristics (Gautam et al., 2020a, 2020b). In several pest and non-pest species, modulations in the insect body AKH levels were observed after insecticide treatment (see Kodrík et al., 2015b).

The co-application of *S. carpocapsae* with Peram-CAH-II caused an increase in *L. decemlineata* mortality compared with the application of the nematode alone (see Fig. 2). This theoretically and practically intriguing ability of AKHs has been observed in various insect species infected with bacteria (Goldsworthy et al., 2005), fungi (Goldsworthy et al., 2005; Mullen and Goldsworthy, 2006; Gautam et al., 2020a), and nematodes (Ibrahim et al., 2017, 2018). Theoretically, there are three possible explanations for this phenomenon:

(A) When AKH is present with an infection, it stimulates general metabolism, which accelerates the turnover of toxins produced by the infection, increasing toxin efficacy. The increasing carbon dioxide production can be used to monitor the rise in metabolism (Ibrahim et al., 2017, 2018; Gautam et al., 2020a, 2020b);

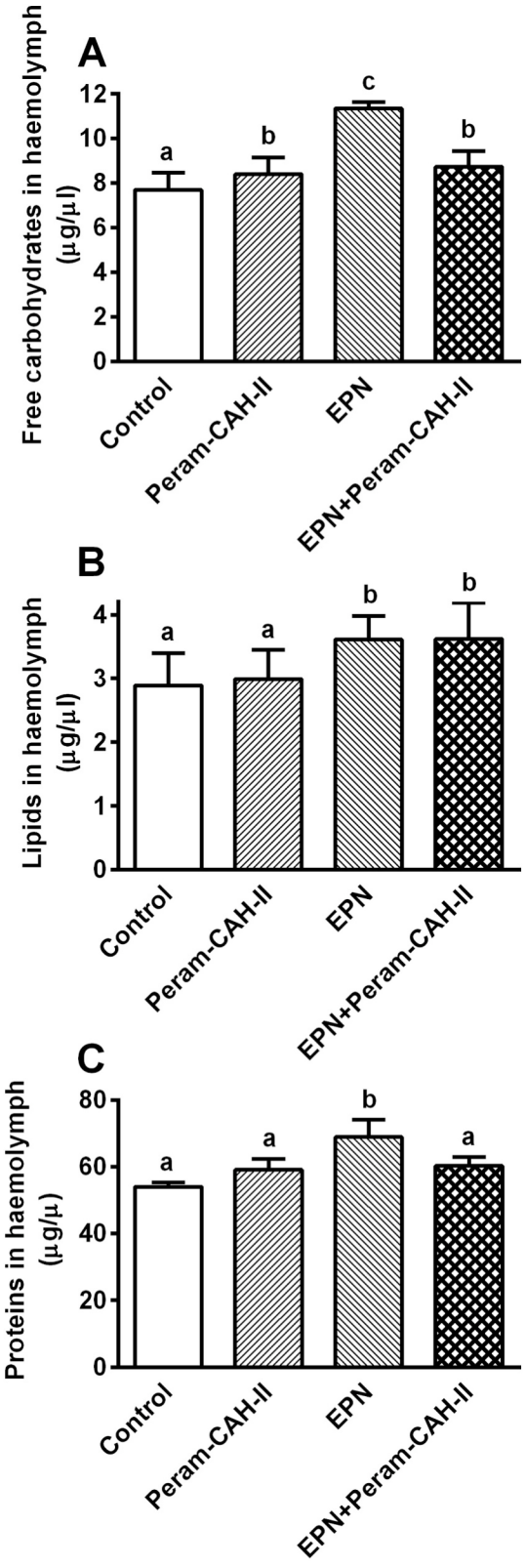

Fig. 7. The effect of injection of 50 individuals of *S. carpocapsae* (EPN) and 40 pmol Peram-CAH-II treatments on level of hemolymph carbohydrates (A), lipids (B), and proteins (C) of *L. decemlineata* 1st day after the treatments. Statistically significant differences among the treatments at 5 % level evaluated by one-way ANOVA with Tukey's post-test are indicated by different letters (a, b, c); bars = mean ± SD, n = 5–10.

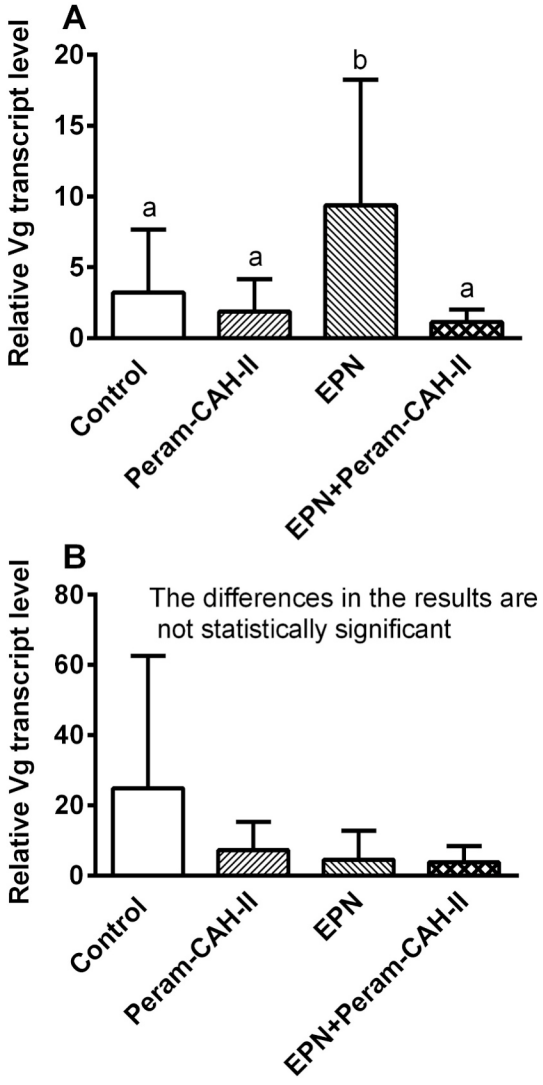

Fig. 8. The effect of injection of 50 individuals of *S. carpocapsae* (EPN) and 40 pmol Peram-CAH-II treatments on relative transcription of vitellogenin (*Vg*) gene in male (A) and female (B) fat bodies of *L. decemlineata* 1st day after the treatments. Statistically significant differences among the treatments at 5 % level evaluated by one-way ANOVA with Tukey's post-test are indicated by different letters (a, b); bars = mean ± SD, n = 10–15.

(B) AKH mobilizes nutrients into the hemolymph, particularly carbohydrates and lipids (Gäde et al., 1997), and supplies energy-rich substrates for pathogen growth (Mullen and Goldsworthy, 2006); and

(C) As AKH has been shown to inhibit RNA (Kodrík and Goldsworthy, 1995) and protein (Carlisle and Loughton, 1979; Moshitzky and Applebaum, 1990; Lorenz, 2003) syntheses, an inhibition of antimicrobial defense proteins (including vitellogenins – see below) in the insect body cannot be ruled out.

4.2. *Effect on metabolism*

In most studies where a pathogen/toxin was combined with AKH, which resulted in a significant increase in mortality, a corresponding increase in metabolism, as measured by carbon dioxide production, was also observed (Kodrík et al., 2010; Velki et al., 2011; Plavšin et al., 2015; Ibrahim et al., 2017, 2018; Gautam et al., 2020a, 2020b). These findings suggest that the mechanism (indicated in Section 4.1 – (A)) of faster toxin turnover could be involved. Surprisingly, when *L. decemlineata* was

treated with *S. carpocapsae* and Peram-CAH-II (in the present study), no such effect was observed. This finding suggests the employment of a different mechanism, most likely the mobilization of metabolites in the insect body for nematode growth (refer to Section 4.1 – (B)). However, the mechanism is not entirely clear, which implies that other factors may be involved. For example, nematode treatment increased the amount of carbohydrates in the fat body; however, when combined with Peram-CAH-II, the level reduced to that in the control. Nevertheless, this phenomenon was not accompanied by carbohydrate release into the hemolymph, since their levels following the nematode+Peram-CAH-II treatment were lower than those in nematode-treated beetles (Figs. 6 and 7). However, it cannot be completely ruled out that this decline could probably be due to more extensive carbohydrate use by rapidly growing nematodes. On comparing the nematode-alone treatment with nematode+Peram-CAH-II treatment, a similar drop in fat body lipid levels was observed, without any drop in the hemolymph. Further, the protein levels in the fat body and hemolymph fluctuated slightly.

In the midgut of *L. decemlineata*, nematode treatment caused no or insignificant changes in digestive enzyme activity. However, after co-application with Peram-CAH-II, a slight decrease in the activity (significant for glucosidases) of enzymes was observed. In general, the role of hormones in the midgut characteristics of *L. decemlineata*, if any, is unclear, although the stimulatory influence on midgut function has been documented in various insect species (Kodrík et al., 2012; Bil et al., 2014; Bodláková et al., 2018).

4.3. Effect on vitellogenin transcription

Vitellogenins (Vgs) are a complex group of proteins that play important roles as yolk protein precursors in egg-laying females. Vgs have also been detected in several insect males (Villar and Grozinger, 2017; Kodrík et al., 2019; Bodláková et al., 2022). This is not surprising, given that Vgs also play roles in other aspects of insect biology, including defense against various entomopathogens (Singh et al., 2013; Salmela and Sundstrom, 2017; Park et al., 2018) or toxins (Bodláková et al., 2022). This phenomenon was also demonstrated in the present study (see Fig. 8), where nematode treatment resulted in a significant increase in Vg transcription in the male CPB fat body. When the firebug *Pyrrhocoris apterus* males were treated with the same nematode, and/or entomopathogenic fungus *Isaria fumosorosea*, a significant increase in *Vg* transcription was observed (Kodrík et al., 2019). However, the effect of nematodes on *Vg* transcription in female fat bodies was not significant in the present study. The decreasing trend in infected females was consistent with the effect of the aforementioned pathogens on *Vg* transcription in *P. apterus* female fat bodies (Kodrík et al., 2019). Although the different effects of nematode infection in male and female fat bodies are startling, this fact need not be perplexing. One might infer that the Vg level required for successful pathogen defense is significantly lower than that required for the nutritional supply of developing eggs. As a result, the female body shuts down less vital processes during infection to save energy for more important tasks. This trade-off strategy is common in insects encountering various stressful situations (Kodrík, 2008; Zhang et al., 2014; Huang et al., 2016).

Peram-CAH-II had a moderate influence on *Vg* transcription, with except for a significant reduction in the high *Vg* transcription level triggered by nematodes (see Fig. 8A – EPN vs. ENP + Peram-CAH-II) in CPB males (item C in Section 4.1). This Vg restriction in the defense system against nematodes could be one reason for the increased CPB mortality caused by Peram-CAH-II; however, this cannot be reliably determined in females. Nonetheless, one of the well-known functions of AKHs is to suppress Vg production (Carlisle and Loughton, 1979; Moshitzky and Applebaum, 1990).

In conclusion, our study demonstrates that the entomopathogenic nematode *S. carpocapsae* causes significant stress in infected adults of the Colorado potato beetle *L. decemlineata*, as evidenced by a rise in AKH level in the body. Furthermore, external co-application of Peram-CAH-II with nematodes considerably increased beetle mortality. The mechanism of action of this phenomenon appears to be complicated and not entirely clear. Increased nematode toxin turnover as a result of increased metabolism in the host body does not appear to be possible. The nematobacterial complex most likely benefits from AKH nutrient mobilization and Vg production, although other unknown components could be involved, complicating the understanding of the mechanism. All these findings are intriguing and could be crucial in developing future pest control strategies, although a complete understanding of the mechanism of action requires further research.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cbpc.2022.109466.

Declaration of competing interest

There are no conflicts of interests to declare.

Data availability

No data was used for the research described in the article.

Acknowledgement

This study was supported by grant No. QK1910270 (OSH) from the National Agency for Agricultural Research, and by projects RVO 60077344 of the Institute of Entomology. The authors thank to Mrs. H. Štěrbová and Mrs. Radka Tanzer Fabiánová for their technical assistance. The English grammar and stylistics were checked by the Editage Author Services.

References

Armer, C.A., Berry, R.E., Reed, G.L., Jepsen, S.J., 2004. Colorado potato beetle control by application of the entomopathogenic nematode Heterorhabditis marelata and potato plant alkaloid manipulation. Entomol. Exp. Appl. 111, 47–58.
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.
Bodláková, K., Černý, J., Štěrbová, H., Guráň, R., Zítka, O., Kodrík, D., 2022. Insect body defence reactions against bee venom: do adipokinetic hormones play a role? Toxins 14, 11.
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.
Čačija, M., Bažok, R., Kolenc, M., Bujas, T., Drmić, Z., Kadoić Balaško, M., 2021. Field efficacy of Steinernema sp. (Rhabditida: Steinernematidae) on the Colorado potato beetle overwintering generation. Plants 10, 1464.
Carlisle, J., Loughton, B.G., 1979. Adipokinetic hormone inhibits protein synthesis in locusta. Nature 282, 420–421.
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.
Chaston, J.M., et al., 2011. The entomopathogenic bacterial endosymbionts Xenorhabdus and Photorhabdus: convergent lifestyles from divergent genomes (53 co-authors). PLoS One 6, e27909.
Duchaud, E., et al., 2003. The genome sequence of the entomopathogenic bacterium Photorhabdus luminescens (25 co-authors). Nat. Biotechnol. 21, 1307–1313.
Dunphy, G.B., Thurston, G.S., 1990. Insect immunity. In: Gaugler, R., Kaya, H.K. (Eds.), Entomopathogenic Nematodes in Biological Control. CRC Press Inc, Boca Raton, FL, pp. 301–323.
Ehlers, R.U., 2001. Mass production of entomopathogenic nematodes for plant protection. Appl. Microbiol. Biotechnol. 56, 623–633.
Ehlers, R.U., 2003. Entomopathogenic nematodes in the European biocontrol market. Commun. Agric. Appl. Biol. Sci. 68, 3–16.
Elpidina, E.N., Vinokurov, K.S., Gromenko, V.A., Rudenskaya, Y.A., Dunaevsky, Y.E., Zhuzhikov, D.P., 2001. Compartmentalization of proteinases and amylases in Nauphoeta cinerea midgut. Arch. Insect Biochem. Physiol. 48, 206–216.
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., Kellner, R., 1989. The metabolic neuropeptides of the corpus cardiacum from the potato beetle and the American cockroach are identical. Peptides 10, 1287–1289.

Gäde, G., Hoffmann, K.H., Spring, J.H., 1997. Hormonal regulation in insects: facts, gaps, and future directions. Physiol. Rev. 77, 963–1032.
Gautam, U.K., Bohatá, A., Shaik, H.A., Zemek, R., Kodrík, D., 2020a. Adipokinetic hormone promotes infection with entomopathogenic fungus Isaria fumosorosea in the cockroach Periplaneta americana. Comp. Biochem. Physiol. C 229, 108677.
Gautam, U.K., Hlávková, D., Shaik, H.A., Karaca, I., Karaca, G., Sezen, K., Kodrík, D., 2020b. Adipokinetic hormones enhance the efficacy of the entomopathogenic fungus Isaria fumosorosea in model and pest insects. Pathogens 9, 801.
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.
Goldsworthy, G.J., Kodrík, D., Comley, R., Lightfoot, M., 2002. A quantitative study of the adipokinetic hormone of the firebug, Pyrrhocoris apterus. J. Insect Physiol. 48, 1103–1108.
Huang, L., Lu, M., Han, G., Dua, Z., 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.
Hussein, H.M., Skoková Habuštová, O., Půža, V., Zemek, R., 2016. Laboratory evaluation of Isaria fumosorosea CCM 8367 and Steinernema feltiae Ustinov against immature stages of the Colorado potato beetle. PLoS One 11, e0152399.
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.
Inman, F.L., Singh, S., Holmes, L.D., 2012. Mass production of the beneficial nematode Heterorhabditis bacteriophora and its bacterial symbiont Photorhabdus luminescens. Indian J. Microbiol. 52, 316–324.
Jolivet, P., 1991. Le doryphore menace l'Asie Leptinotarsa decemlineata Say 1824 (Col. Chrysomelidae). Entomologiste 47, 29–48.
Kodrík, D., 2008. Adipokinetic hormone functions that are not associated with insect flight. Physiol. Entomol. 33, 171–180.
Kodrík, D., Socha, R., 2005. The effect of insecticide on adipokinetic hormone titre in insect body. Pest Manag. Sci. 61, 1077–1082.
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., 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., 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., 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., 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., 2015. 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., 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., Ibrahim, E., Gautam, U.K., Čapková-Frydrychová, R., Bednářová, A., Krištůfek, V., Jedlička, P., 2019. Changes in vitellogenin expression caused by nematodal and fungal infections in insects. J. Exp.Biol. 222.
Krishnan, N., Večeřa, J., Kodrík, D., Sehnal, F., 2007. 20-Hydroxyecdysone prevents oxidative stress damage in adult Pyrrhocoris apterus. Arch. Insect Biochem. Physiol. 65, 114–124.
Lorenz, M.W., 2003. Adipokinetic hormone inhibits the formation of energy stores and egg production in the cricket Gryllus bimaculatus. Comp. Biochem. Physiol. B 136, 197–206.
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., Applebaum, S.W., 1990. The role of adipokinetic hormone in the control of vitellogenins in locusts. Insect Biochem. 20, 319–323.
Mochanová, M., Tomčala, A., Svobodová, Z., Kodrík, D., 2018. Role of adipokinetic hormone during starvation in Drosophila. Comp. Biochem. Physiol. B 226, 26–35.
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.
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.
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. Comp. Biochem. Physiol. C 170, 19–27.
Půža, V., Nermuť, J., Konopická, J., Skoková, Habuštová O., 2021. Efficacy of the applied natural enemies on the survival of Colorado potato beetle adults. Insects 12, 1030.
Roberts, I.M., 1985. Hydrolysis of 4-methylumbelliferyl butyrate: a convenient and sensitive fluorescent assay for lipase activity. Lipids 20, 243–247.
Salmela, H., Sundstrom, L., 2017. Vitellogenin in inflammation and immunity in social insects. Inflamm. Cell Signal. 4, e1506.
Shi, X.Q., Guo, W.C., Wan, P.J., Zhou, L.T., Ren, X.L., Ahmat, T., Fu, K.Y., Li, G.Q., 2013. Validation of reference genes for expression analysis by quantitative real-time PCR in Leptinotarsa decemlineata (Say). BCM Res. Notes 6, 93.
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.
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.
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: Deutscher, M.P. (Ed.), Methods in Enzymology, vol. 182. Academic Press, London, pp. 50–68.
Thurston, G.S., Dunphy, G.B., Yule, W.N., 1994. Explanations for the low susceptibility of Leptinotarsa decemlineata to Steinernema carpocapsae. Biol. Control 4, 53–58.
Trdan, S., Vidrih, M., Andjus, L., Laznik, Ž., 2009. Activity of four entomopathogenic nematode species against different developmental stages of Colorado potato beetle, Leptinotarsa decemlineata (Coleoptera, Chrysomelidae). Helminthologia 46, 14–20.
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.
Villar, G., Grozinger, C.M., 2017. Primer effects of the honeybee, Apis mellifera, queen pheromone 9-ODA on drones. Anim. Behav. 127, 271–279.
Wang, Y., Gaugler, R., Cui, L., 1994. Variations in immune response of Popillia japonica and Acheta domesticus to Heterorhabditis bacteriophora and Steinernema species. J. Nematol. 26, 11.
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 VO2, VCO2 and evaporative water loss with a flow-through mask. J. Appl. Physiol. 42, 120–123.
Zhang, W.N., Xiao, H.J., Liang, G.M., Guo, Y.Y., 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.
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.