# ULOGA ADIPOKINETIČKOG HORMONA U OKSIDATIVNOM STRESU UZROKOVANOM OTROVOM BRAKONIDNE OSE HABROBRACON HEBETOR

**Kljajić, Kristina**

**Master's thesis / Diplomski rad**

**2018**

*Degree Grantor / Ustanova koja je dodijelila akademski / stručni stupanj:* **Josip Juraj Strossmayer University of Osijek, Department of biology / Sveučilište Josipa Jurja Strossmayera u Osijeku, Odjel za biologiju**

*Permanent link / Trajna poveznica:* https://urn.nsk.hr/urn:nbn:hr:181:420088

Josip Juraj Strossmayer University of Osijek
Department of Biology
Graduate university study programme in Biology

Kristina Kljajić

# Role of adipokinetic hormone in oxidative stress elicited by venom from the braconid wasp *Habrobracon hebetor*

Master's Thesis

Osijek, 2018.

**TEMELJNA DOKUMENTACIJSKA KARTICA**

**Sveučilište Josipa Jurja Strossmayera u Osijeku** **Diplomski rad**

**Odjel za biologiju**

**Diplomski sveučilišni studij Biologija; smjer: znanstveni**

**Znanstveno područje:** Prirodne znanosti

**Znanstveno polje:** Biologija

# ULOGA ADIPOKINETIČKOG HORMONA U OKSIDATIVNOM STRESU UZROKOVANOM OTROVOM BRAKONIDNE OSE *HABROBRACON HEBETOR*

**Kristina Kljajić**

**Rad je izrađen:** Laboratorij za fiziologiju kukaca, Institut za entomologiju, České Budějovice
**Mentor:** doc. dr. sc. Nataša Turić
**Komentor**: prof. dr. sc. Dalibor Kodrík

**Kratak sažetak diplomskog rada:** Glavni cilj rada je bio istražiti navodnu ulogu adipokinetičkog hormona u antioksidativnom odgovoru kod kukca *Pyrrhocoris apterus* tretiranog otrovom *Habrobracon hebetor*. Praćeni su biomarkeri oksidativnog stresa: sadržaj protein karbonila te aktivnosti glutation S-transferaze i katalaze. Kvantificiran je ukupan sadržaj adipokinetičkog hormona u središnjem živčanom sustavu. Ubrizgavanje otrova u plamenu stjenicu uzrokovalo je smanjenje sadržaja protein karbonila u hemolimfi ali je povećalo karbonilaciju probavila. Ko-tretmanom otrova i Pyrap-AKH sadržaj protein karbonila u hemolimfi se vratio na kontrolnu razinu ali se povećao iznad kontrolne razine u probavilu. Otrov je uzrokovao povećanje aktivnosti glutation S-transferaze u probavilu i umanjio aktivnost katalaze. Nakon ko-tretmana aktivnosti glutation S-transferaze i katalaze nisu se promjenile u odnosu na tretman otrovom što je neočekivano. Kvantifikacija Pyrap-AKH pokazala je povišene razine adipokinetičkog hormona u središnjem živčanom sustavu nakon ubrizgavanja otrova. Mišići tjelesne stjenke nisu reagirali na djelovanje otrova. Očito je da otrov uzrokuje oksidativni stres kod plamene stjenice ali rezultati nakon ko-tretmana otrova i adipokinetičkog hormona nisu potvrdili očekivanja.

**Broj stranica: 37**
**Broj slika: 10**
**Broj tablica: 0**
**Broj literaturnih navoda: 66**
**Jezik izvornika: engleski**

**Ključne riječi:** *Pyrrhocoris apterus,* biomarkeri, probavilo, hemolimfa, središnji živčani sustav

**Datum obrane:** 31.10.2018.

**Stručno povjerenstvo za obranu:**

1. Dr.sc. Goran Vignjević, doc., predsjednik
2. Dr.sc. Nataša Turić, doc., član
3. Dr.sc. Stjepan Krčmar, prof., član
4. Dr.sc. Sandra Ečimović, doc., zamjena člana

**Rad je pohranjen:** na mrežnim stranicama Odjela za biologiju te u Nacionalnom repozitoriju završnih i diplomskih radova Nacionalne i sveučilišne knjižnice u Zagrebu

**BASIC DOCUMENTATION CARD**

**Josip Juraj Strossmayer University of Osijek** **Master's Thesis**

**Department of Biology**

**Graduate university study programme in Biology**

**Scientific area:** Natural science

**Scientific field:** Biology

# ROLE OF ADIPOKINETIC HORMONE IN OXIDATIVE STRESS ELICITED BY VENOM FROM THE BRACONID WASP *HABROBRACON HEBETOR*

**Kristina Kljajić**

**Thesis perfomed at:** Department of Insect physiology, Institute of Entomology, České Budějovice
**Supervisor:** Nataša Turić, PhD, Assistant Professor
**Cosupervisor:** Dalibor Kodrík, PhD, Associate Professor

**Short abstract:** The main goal of the study is to explore this putative role of adipokinetic hormone in anti-oxidative stress response in the firebug *Pyrrhocoris apterus* body poisoned by *Habrobracon hebetor* venom. Oxidative stress biomarkers were monitored: protein carbonyl content and activity of glutathione S-transferase and catalase. Adipokinetic hormone level was quantified in central nervous system. Injection *Habrobracon hebetor* venom into *Pyrrhocoris apterus* body caused decrease in protein carbonyl content in hemolymph but increased carbonylation in midgut. When co-treated with venom and Pyrap-AKH, protein carbonyls returned to control level in hemolymph but increased above control level in midgut. Venom elicited activity of glutathione S-transferase in midgut but decreased catalase activity. After co-treatment glutathione S-transferase and catalase activity did not change compared to venom treatment which was not expected. Quantification of Pyrap-AKH showed elicited adipokinetic level in central nervous system after venom injection. Body wall muscles were not affected by venom. It is apparent that venom elicited oxidative stress in *P. apterus* but co-application of venom and adipokinetic hormone did not match the expectation.

**Number of pages: 37**
**Number of figures: 10**
**Number of tables: 0**
**Number of references: 66**
**Original in: English**

**Keywords:** *Pyrrhocoris apterus,*biomarkers, midgut, hemolymph, central nervous system

**Date of thesis defence:** 31.10.2018.

**Reviewers:**

1. Goran Vignjević, PhD, assistent professor, chair
2. Nataša Turić, PhD, assistent professor, member
3. Stjepan Krčmar, PhD, full professor, member
4. Sandra Ečimović, PhD, assistent professor, member

**Thesis deposited in:** on the Department of Biology website and the Croatian Digital Theses Repository of the National and University Library in Zagreb.

*Acknowledgments*

*I would first like to thank my supervisor doc. dr. sc. Nataša Turić for her guidance, constructive comments and helpful suggestions which improved this thesis.*

*I would like to express my sincerest appreciativeness to my co-supervisor prof. Dalibor Kodrík from Department of Biochemistry and Physiology at the Institute of Entomology in České Budějovice on the opportunity to do my experimental work in the department. His guidance and suggestions were very helpful in making of my thesis.*

*I would also like to acknowledge the staff of Laboratory of Insect Physiology, particularly to Haq Abduk Shaik for his patience, advices and availability whenever I needed help during my work in the laboratory.*

*Finally, I express my gratitude to my family for providing me with support and continuous encouragement throughout my years of study.*

TABLE OF CONTENT

# 1. INTRODUCTION

A minute Braconidae wasp *Habrobracon hebetor* is a polyphagous and gregarious parasitoid that can parasitize larvae or adults of various insects. The *H. hebetor* venom elicits complete neuromuscular paralysis in insects and strongly suppresses the cell and humoral immunity (Kryukova *et al.,* 2007). The main reasons of those activities is immobilization of the host ('a living can') and suppression of its defense immune response, which provide optimal living conditions for the laid eggs and a new generation of the wasp. The venom consists of a complex cocktail of proteinaceous and non-proteinaceous components that affect various biochemical reactions and physiological processes in the body of attacked insects (Abdul *et al.,* 2017). Interestingly, till date, despite a large number of the parasitic wasp species only few types of venom with biological activity were identified and functionally evaluated.

## 1.1. Insects as model organisms

Microorganisms, plants or animals used for scientific research are called model organisms. Main idea was that certain organisms can be used to gain knowledge about other organisms. Model organisms should be able to breed easily (or multiply), should be widely distributed and suitable for maintaining in lab or culture. Its biology should be well known and it should be sensitive to factors that are being researched.

Insects are good model organisms. There are more than million species and their diversity and distribution is remarkable. They also live in urban and agricultural areas where they are exposed to numerous chemicals. Some of them are parasites and some are very useful as pollinators or food.

Insects are widely used in scientific research (drosophila, cockroaches, locusts, beetles). Some of the most significant genetic studies in 20$^{th}$ century involved *Drosophila melanogaster* including the study of Thomas Hunt Morgan and his chromosomal theory of heredity (Morgan, 1911). Morgan's work on *D. melanogaster* led to the development of first genetic map, the discovery of X-ray induced mutagenesis, discovery of Hox genes and many more. In last 40 years, *D. melanogaster* has been used to understand regenerative

biology, especially pathway of stem cells. The red flour beetle, *Tribolium castaneum*, has been used in understanding how vertebrates determine their body plans during development. Development of *T. castaneum* can be manipulated with RNA interference. Focus was on Wnt genes which helped the developing beetle body determine which end will be head and which will be the rear (Bolognesi *et al.,* 2008). By studying Wnt pathway in *T. castaneum* and similar model organisms, scientists can better understand mechanism of this pathway and follow its evolution in more complex organisms. Firebug, *Pyrrhocoris apterus* (Heteroptera) is also used for research in insect neuroendocrinology and physiology during last five decades. The emphasis is on diapause and its regulation, particularly seasonal switch depending on day-length measurement (Hodkova and Hodek, 2004) and neurohormones and their receptors which are promising targets for controlling pest species (Gäde and Goldsworthy, 2003). Breeding of this specie in a lab is easy and there are lot of information about its behavior, genetics, morphology, physiology and biochemistry. Firebug *P. apterus* is used in this research as model organism.

### 1.1.1. Central nervous system and endocrine system

Central nervous system (CNS) is made of series of ganglia. There are three main ganglia in the head: protocerebrum, deuterocerebrum and tritocerebrum. These ganglia are fused together and make a brain or supraesophageal ganglion. Rest of ganglionic chain is located on the ventral side of the body. Brain is connected to the subesophageal ganglion (Web 1). CNS and endocrine system are closely linked. Endocrine system is made of specialized endocrine glands and neurosecretory cells mostly localized in the CNS (Figure 1.)

Endocrine glands:

1. glands producing ecdysteroids
2. prothoracic gland
3. corpora allata
4. corpora cardiaca
5. endocrine cells of the midgut

Corpora cardiaca (CC) store and release hormones from the neurosecretory cells of the brain and (among others) produce AKHs. Corpora allata (CA) produce juvenile hormone which regulates metamorphosis. CA is connected to CC (Chapman, 1969).

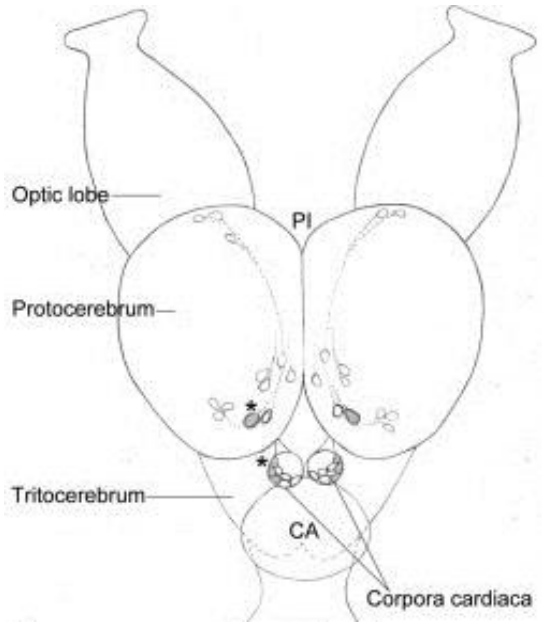


Figure 1. Schematic representation of *P. apterus* brain along with CC and CA
(After Kodrik *et al*., 2015.)

### 1.1.2. Adipokinetic hormones (AKHs)

Adipokinetic hormones are neuropeptides involved in metabolism and generation of energy. They are synthesized, stored and released by neurosecretory cells from CC. By this date, over 40 insect AKHs have been described. These hormones are pleiotropic with numerous activities related to their metabolic role. They stimulate catabolic reactions (mobilize lipids, carbohydrates and/or certain amino acids) and in the same time inhibit synthetic reactions thus acting like typical stress hormones. AKHs consist of 8 – 10 amino acids. N-terminus is blocked by pyroglutamate residue and amide group is blocking C-terminus.

Numerous AKHs have been described from all major insect orders (Gäde *et al.,* 1997; Gäde *et al.*, 2003) including Heteroptera with several different AKHs characterized (Kodrik *et al.*, 2010). Firebug *Pyrrhocoris apterus* is often used for research of AKH. Two AKHs have been described in this specie: Pyrap-AKH (pGlu-Leu-Asn-Phe-Thr-Pro-Asn-Trp-$NH_2$) and Peram-CAH-II (pGlu-Leu-Thr-Phe-Thr-Pro-Asn-Trp-$NH_2$) (Kodrik *et al.,* 2000; Kodrik *et al.*, 2002).

### 1.1.3. Properties of *Habrobracon hebetor* venom

The venom of parasitic wasps is a complex cocktail of proteinaceous and non-proteinaceous components that affect various biochemical reactions and physiological processes in the body of the attacked insects. Braconidae wasp *Habrobracon hebetor* is a polyphagous and gregarious specie that primarily parasitizes lepidopteran and coleopteran larvae (Benson, 1974; Kovalenkov, 1984; Nay and Perring, 2005; Altuntas *et al*., 2010). Venom elicits complete neuromuscular paralysis in insects (Beckage and Gelman, 2004; Sláma and Lukáš, 2011) and strongly suppresses cell and humoral immunity (Kryukova *et al*., 2007, 2011). It affects the properties of hemocytes and induces $Ca^{2+}$ release from intracellular stores via PLC activation that leads to cell death (Kryukova *et al.* 2005).Venom does not affect peripheral inhibition (heart, gut) (Piek *et al.* 1970, 1974). There may be a specific affinity of the venom to glutaminergic synapses (Walther *et al*., 1974) and its components interact with receptors on presynaptic site (Pennachio, Strand, 2006). In Lepidoptera and locusts the venom presynaptically blocks the excitatory but not inhibitory neuromuscular transmission (Piek *et al*., 1982). It inhibits exocytosis of presynaptic vesicles (Piek, 1966; Walther, 1983).

Several neurotoxins were isolated from the venom: A-MTX and B-MTX (Visser *et al*., 1983), Brh-I, Brh-III, Brh-V (Quisted *et.al.*, 1994), BrhTx-1, BrhTx-2 (Windass *et al*., 1996). Structure of paralyzing toxin BrhTx-1 is known: 4 subunits, 3 of them share sequence identities either with phospholipases or with family of chymotrypsin –like proteases from various organisms (Moreau, Guillot 2005). Altuntas *et al*. (2008) investigated post-parasitism effect of *H. hebetor* on hemolymph plasma proteins in the final instar larvae of *Ephestia kuehniella*. They found only quantitative changes in host plasma proteins. It didn't lead to up regulation of novel proteins and some proteins were broken down or immuno suppressed. Level of 24, 6 and 19 kDa protein bands increased which may be defensive proteins.

## 1.2. Oxidative stress

Oxidative stress can be defined as impairment in production of reactive oxygen species (ROS) and body's natural anti-oxidative defense. That kind of imbalance can lead to carbonylation of proteins, lipid peroxidation and oxidative damage to DNA which can result with various disorders or even death (Figure 2.)

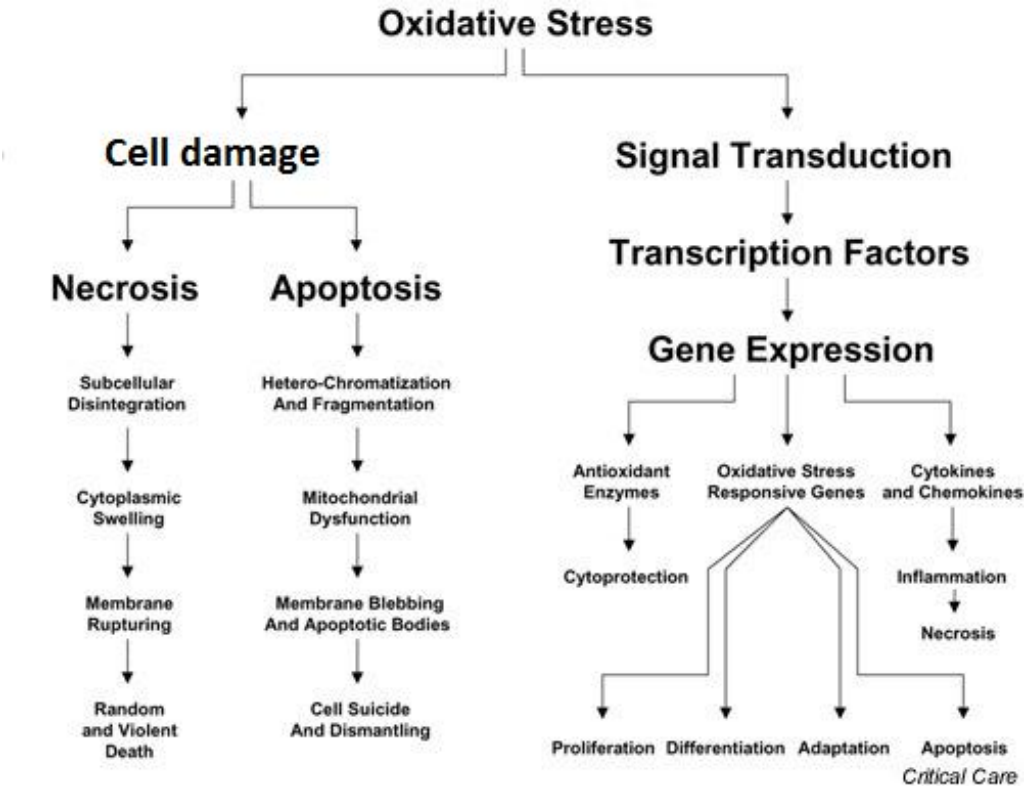


Figure 2. A general schematic showing the regulation of cellular processes in response to oxidative stress (Modified after Haddad, 2002)

ROS are generated by living organisms during normal cellular metabolism. They can occur in two forms: as free radicals and non-radical. Free radicals are produced when molecules have one or more unpaired electrons which make them reactive. Non-radical forms are produced when two free radicals share their unpaired electrons. Once radicals form, they can react with another radical or with another molecule by various interactions. The rate and selectivity of these types of reactions depend on high concentration of radicals, on

delocalization of the single electron of the radical and on the absence of weak bonds in any other molecules present with which the radical could interact.

The 3 major ROS that are of physiological significance are superoxide anion ($O^{2-}$.), hydroxyl radical (•OH) and hydrogen peroxide ($H_2O_2$).

Superoxide radical is considered to be „primary“ ROS. It is produced by one-electron reduction of oxygen and can interact with other molecules to produce „secondary“ ROS. Superoxide radical also acts like a mediator in oxidative chain reactions. Dismutation of this radical can occur in two ways: spontaneously or through a reaction catalyzed by superoxide dismutases. Product of such dismutation is hydrogen peroxide ($H_2O_2$). It can be fully reduced to water or partially reduced to hydroxyl radical (•OH). Hydroxyl radical is very potent and highly reactive: it can damage all types of macromolecules: nucleic acids, lipids, carbohydrates and amino acids. Unlike superoxide, it cannot be eliminated by enzymatic reactions (Reiter *et al.*, 1995). Free radicals can be produced under both normal and pathological conditions. While high concentrations of ROS cause cell death, at low concentrations they can initiate cell proliferation. Low concentration of superoxide radical and hydrogen peroxide stimulate proliferation and enhanced survival in wide variety of cell types. Thus, ROS play important role as second messengers (Lowenstein *et al.*, 1994).

Other examples include regulation of the cytosolic calcium concentration, regulation of protein phosphorylation, and activation of certain transcription factors (Storz, 2005).

There are varieties of antioxidants that serve to counterbalance the effect of oxidants. It can be divided into two groups: enzymatic and non-enzymatic. Antioxidant enzymes include catalase (CAT), superoxide dismutase (SOD), GSTs and AsPx.

### 1.2.1. Oxidative stress biomarkers

The definition of a biomarker according The National Institute of Health is a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes or pharmacological responses to a therapeutic intervention (Zhang *et al.,* 2001).

Oxidative stress biomarkers are molecules which are modified by interactions with ROS and molecules of antioxidant system that change in response to increased redox stress (Ho *et al.,* 2013). By measuring activity of antioxidative enzymes and antioxidants, it is possible to evaluate oxidative stress.

### 1.2.2. Protein carbonyls

ROS can promote protein oxidation known as protein carbonylation. It is a process where reactive ketones or aldehydes are formed. They can react with 2,4-dinitrophenylhydrazine (DNPH) to form hydrazones. This then can be detected by various means, such as spectrophotometric assay, enzyme-linked immunosorbent assay (ELISA), and one-dimensional or two-dimensional electrophoresis followed by Western blot immunoassay. There are primary and secondary protein carbonylation. In primary protein carbonylation side chains of lysine, arginine, proline and threonine residues are susceptible to direct oxidation which produces DNPH detectable protein products (Levine, 2002; Stadtmann and Levine, 2000; Butterfield and Stadtmann, 1997). In secondary protein carbonylation DNPH detectable protein products are formed by adding aldehydes (for example from lipid peroxidation) (Grimsrud *et al.,* 2008; Sayre *et al.,* 2006).

Quantitatively, most important products of carbonylation are glutamic semialdehyde of arginine and proline and aminoadipic semialdehyde of lysine (Requena *et al.,* 2003).

Relative early formation of protein carbonyl and its stability are main advantages when using them as oxidative stress biomarker. Also no special or expensive equipment is required.

### 1.2.3. Glutathione S-transferase

Glutathione S-transferases are multifunctional enzymes of phase II detoxification. There are three superfamilies: cytosolic, mitochondrial and microsomal (MAPEG). They play key role in cellular detoxification by conjugating toxicants to glutathione making products more water soluble by neutralizing their electrophilic site (Boyland and Chasseaud, 1969). Other functions include peroxidase and isomerase activities, protecting cells against $H_2O_2$ and binding non-catalytically a wide range of endogenous and exogenous ligands.

Lately, GST activity in insects is being under intensive research for its role in development of insecticide resistance (Che-Mendoza *et al.*, 2009).

### 1.2.4. Catalase

Catalase is enzyme present in peroxisomes of aerobic cells. It protects them from effects of hydrogen peroxide by catalyzing its degradation where molecular oxygen and water are formed. Catalase is made of four subunits and every subunit has heme prosthetic group in catalytic center. It is stable and more resistant to pH, proteolysis and denaturation than other enzymes. Regulation of CAT depends on cell's oxidative state while it is one of enzymes that form first line of defense against free radicals. Other factors are included in regulation of CAT such as hormones (melatonin, growth hormone and prolactin).

## 1.3. Review of recent studies of AKH and *Pyrrhocoris apterus* as model species

There are various stressors that can disrupt homeostasis and result in stress situation. Stressors can be physical (temperature, radiation, noise, etc.), physiological (aging, injury, diseases, etc.) and chemical (metals, drugs, pesticides, etc.).

Oxidative stress is state in which homeostasis is disrupted by enhanced production of reactive oxygen species (ROS). ROS can damage proteins, nucleic acids and lipids. To prevent serious damage, defense systems are developed. In insects such defense system is regulated by adipokinetic hormones (AKHs) secreted from corpora cardiaca – neuroendocrine gland connected to the brain.

One of the excellent models where AKH functions in insect body are intensively studied is the firebug *Pyrrhocoris apterus*; its two AKHs: Pyrap-AKH (pGlu-Leu-Asn-Phe-Thr-Pro-Asn-Trp-$NH_2$) and Peram-CAH-II (pGlu-Leu-Thr-Phe-Thr-Pro-Asn-Trp-$NH_2$) are well characterized including a sequence of their cDNA encodings and amino acid composition of their pre-pro-hormones.

*P. apterus* is distributed in Mediterranean area, East and Central Asia. In Europe, this insect is fed on seeds of *Tilia* spp.

Firebugs have polymorphed wings and have lost ability to fly (Honek, 1995; Socha and Zemek, 2000). Wings can be long (macroptera) and short (brachyptera) (Socha, 1993). Size of wings and diapause are controlled by temperature and photoperiod. When photophase is shorter than 16h, insects are in diapause and all individuals are brachypteras. Macropteras are developed when photophase is longer than 16h and temperature is increased (Hodek, 1968; Honek, 1976; Honek, 1981).

Beside morphological differences between brachypteras and macropteras, there are also physiological and behavioral differences: macropteras have reduced activity of digestive enzymes and feeding rate (Socha *et al.*, 1998; Šula *et al.*, 1998), stronger AKH response (Socha *et al.*, 1999a) etc.

AKH of *P. apterus* was not yet identified when research on firebugs started. Experiments were performed on macropteras and brachypteras to study differences in AKH characteristics and for that purpose was used AKH of *Locusta migratoria* called Locmi-AKH-I. When Locmi-AKH-I was injected, lipids in hemolymph increased. Adipokinetic response was determined in relation to age, wing dimorphism and reproductive arrest (Socha *et al.*, 1999a).

Socha *et al.* (1999b) wanted to know if AKH can affect locomotor movements of *P. apterus*. Experiments were performed on females and their activity was monitored after AKH and Ringer injections (control). Locomotor movement was increased in insects injected with AKH. Two AKHs in firebugs were described: Pyrap-AKH and Peram-CAH-II (Kodrik *et al.*, 2000; Kodrik *et al.*, 2002b).

Quantitative study of *P. apterus* AKH was performed on nervous system organs and it showed measurable Pyrap-AKH level in CC, 4 pmol per insect (Kodrik *et al*., 2000). AKH

in hemolymph was possible to measure using ELISA after preliminary purification of hemolymph.

Kodrik *et al.* (2002a) described effects of topical application of Pyrap-AKH on locomotor movement and injection of same hormone and possible stress after injection. Both topical application and injection showed elevated lipids in hemolymph and stimulated locomotor movement. Positive correlation between hyperlipidemia and increased locomotor movement indicated that stress factor induced by injection could be connected with adipokinetic response.

To explore possibility that AKHs have important role in other stress situations, effect of permethrin insecticide was researched in CNS and hemolymph of *P. apterus* (Kodrik *et al.*, 2005). In hemolymph of macropteras, level of AKH was 6-8 time higher, in reproductive brachypteras 2,5 times higher and in brachypteras in diapause no increase was detected.

In following study, effect of interactions between AKH and pyrethroid insecticide on physiological processes in *P. apterus* was researched (Kodrik *et al.*, 2010a). Co-treatment of Pyrap-AKH and insecticide increased mortality compared to insecticide treatment only. Injection of insecticide increased AKH level in CNS and hemolymph. Increased effect of insecticide after AKH co-treatment is probably result of stimulatory effect on metabolism – production of $CO_2$ was increased.

Possible involvement of AKH in neural/neuromuscular signaling and defense against stress elicited by neuromuscular paralysis was explored. *Habrobracon hebetor* venom was used to induce paralysis in adult *P. apterus* females. Venom application elicited neuromuscular paralysis and co-application with Pyrap-AKH reduced paralysis up to 3 times compared to venom only. Venom application increased level of AKHs in CNS and hemolymph and expression of both *Akh genes*. Total metabolism declined after venom application (Abdul *et al.*, 2017).

### 1.4. Research goal

The main goal of the study is to explore this putative role of AKH in anti-oxidative stress response in the firebug *P. apterus* body poisoned by *H. hebetor* venom. It is known that external application of AKH can reduce/eliminate oxidative stress and it is supposed that application of the *H. hebetor* venom elicits oxidative stress in the host body.

# 2. MATHERIALS AND METHODS

## 2.1. Experimental insects

### 2.1.1. Classification: Firebug *Pyrrhocoris apterus* (Linnaeus, 1758)

Kingdom: *Animalia*
Phylum: *Arthropoda*
Class: *Insecta*
Order: *Hemiptera*
Suborder: *Heteroptera*
Infraorder: *Pentatomomorpha*
Superfamily: *Pyrrhocoroidea*
Family: *Pyrrhocoridae*
Genus: *Pyrrhocoris*
Species: *P. apterus*

### 2.1.2. Biology of firebug *P. apterus*

Firebug is common insect in Pyrrhocoridae family. It has red and black coloration and is widely distributed throughout Palearctic from the Atlantic coast of Europe to northwest China. It has also been reported from the USA, Central America and India. They are frequently observed to form aggregations, especially immature forms. They feed on linden seeds.

Firebugs generally mate in April and May and have simple metamorphosis which means their life cycle has three stages: egg, nymphs and adults. They go through 5 nymph instar stages (as do most Hemiptera), where they resemble the adults more and more as they grow. Only adults have wings. Firebugs are dimorph species – adults can have long wings (macroptera) or short wings (brachyptera) (Seidenstücker, 1953). Most of them are short - winged, although few of long - winged individuals can appear in every population (up to 14%). This specie appears on the edges of the forest, on poorly grassy and bare surfaces with convenient hibernation locations.

### 2.1.3. Laboratory cultures

Laboratory stock cultures of the firebug *Pyrrhocoris apterus* L. (Heteroptera: Pyrrhocoridae) originating from the wild populations at Ceske Budejovice, Czech Republic were used for experiments. All stages, from egg to adult, were reared under a long-day (LD 18:6h) photocycle at a temperature of 26±1 °C, allowing continuous breeding of the bugs. Larvae and adults were supplied with linden seeds and water *ad libitum*. Freshly ecdysed adults were transferred to small 0.25 L glass jars (females and males separately) and were kept under the same photoperiodic, food and temperature regimes as those under which they had developed. To avoid possible complications from the ovarian cycle, only adult males (7 days old) were used for experiments. Overall 24 insects were used for every experiment – 6 insects in each treated group.

### 2.1.4. Classification: *Habrobracon hebetor* (Say, 1836)

Kingdom: *Animalia*
Phylum: *Arthropoda*
Class: *Insecta*
Order: *Hymenoptera*
Family: *Braconidae*
Genus: *Habrobracon*
Species: *H. hebetor*

### 2.1.5. Laboratory cultures

Individual flask containing *H. hebetor* were reared under the controlled conditions as described for *P. apterus*. Live female wasps were collected into tubes and frozen (−20 °C). As needed, they were retrieved, kept on ice and the venom glands were dissected under a dissection microscope. The dissected glands were washed with Ringer saline, and placed into a tube with a fresh portion of Ringer saline (600 µl) on ice.

Glands were sonicated and filtrated through 0,22 µm filter. Venom was divided in tubes, each tube containing 10 glands in 95,2 µl Ringer saline and stored in freezer (-20°C).

### 2.2.Treatments

Venom extract 0.2 gland equivalent in 2 µl volume of Ringer saline was injected with help of a Hamilton syringe through the metathoracal – abdominal intersegmental membrane to the thorax of experimental 7-days old *P. apterus* males. Controls were injected the same way with the 2 µl of Ringer saline.

A dose of 80 pmol Pyrap-AKH was applied topically as described previously (Kodrík *et al*., 2002): 2 µl of the hormonal solution in 20% methanol in Ringer saline was applied using a pipette onto the thorax and abdomen under the wings. Co – treatment with venom and hormone was performed also.

### 2.3. Dissection

#### 2.3.1. Midgut

Insects were dissected under microscope in 50 mM PBS solution (pH=7). Wings were cut off using scissors. Midgut was carefully removed with pincettes and cleaned from fat body. Weight was noted and placed in tube, one midgut in one tube. Every treatment had 3 biological replicates and was repeated 3 times. It was stored in tubes until further use (-80°C).

#### 2.3.2. Body wall muscles

Insects were also dissected under microscope. Head, wings and legs were cut off with scissors. Gut, fat body, Malpighian tubules and gonads were removed and body wall with muscles were stored (-80°C).

#### 2.3.3. Central nervous system

Dissection of central nervous system was performed under the microscope. Using scissors and tweezers, insect's head with first thoracal segment was cut off. The thoracal segment was removed along with other organs. To remove adipose tissue, head was washed with Ringer solution and brain (including CC and CA) was removed and stored until further use.

### 2.4. Hemolymph extraction

Insect's antennae were cut off and its body was gently pressed. Clear hemolymph was collected on filter paper and transferred in tubes with micropipette. In tubes were previously added granules of thio urea. Each tube contained 1 µl of hemolymph from one insect and 50 mM PBS (pH=7) was added.

### 2.5. AKH extraction from CNS

Methanol extraction was used to extract AKH from central nervous system (brain + CC and CA). In every sample (one CNS) was added 100 µl of 80% methanol and sonicated. The samples were centrifuged for 5 min at 10 000 g and supernatants were collected. The step was repeated one more time. Another 200 µl of 80% MetOH was added into the pellets to wash off material on tube wall. Samples were centrifuged for 5 min at 10 000 g and supernatants were collected again. Supernatants were mixed and evaporated and pellet residues were discarded. After the evaporation, samples were resuspended in buffer and used to determine AKH using competitive ELISA.

### 2.6. Quantification of AKH using competitive ELISA

To determine total content of AKH in CNS of *Pyrrhocoris apterus*, competitive ELISA was used. In microtiter plate was added coating buffer, 100 µl per well, which contained IgG diluted 1:2000. The plate was stored on 4°C over night. The wells were washed 3 times with 200 µl washing buffer and then blocked with 5% milk in washing buffer. The plate was incubated 2 hours on 37°C. Washing process was repeated 3 times. After blocking, samples were added to the wells and incubated again for one hour on 37°C. After washing, horseradish peroxidase (HRP) was applied diluted in washing buffer 1:500 and incubated for same amount of time. Wells were washed 6 times and OPD substrate was added. Samples were incubated in dark for 40 min and temperature of 37°C. Reaction was stopped by adding 0,5 M $H_2SO_4$. Absorbance was read in ELISA reader on 492 nm.

## 2.7. Oxidative stress

### 2.7.1. Protein carbonyls in hemolymph, midgut and body wall muscles

Total protein carbonyls were quantified after their reaction with 2,4-dinitrophenylhydrazine (DNPH) (Levine *et al*., 1990). Carbonyls were quantified spectrophotometrically at 370 nm in a microtitre plate reader. The results were expressed as nmol/mg protein using an extinction coefficient of 22 000 $M^{-1}cm^{-1}$. A bovine serum albumin standard curve was used to quantify protein concentrations in guanidine solutions measured at 280 nm. Protein carbonyl values were corrected for interfering substances by subtracting the A370/mg protein measured without DNPH (controls). Experiments were performed 4 and 24 hours after the treatments.

**Hemolymph**

One microliter of hemolymph was diluted in 150 µl of PBS. Samples were vortexed and centrifuged on 8000 g for 10 min. Supernatants were collected in clean tubes which contained 500 µl of PBS (pH 7). Streptomycin sulfate was added and samples were incubated at room temperature for 15 min. Every sample was divided in two tubes: one containing DNPH and one containing 2M HCl (controls). All samples were incubated in dark for one hour on 37°C. In each tube was added 28% TCA and then centrifuged for 10 min on 15 000 g. Supernatants were discarded and 5% TCA was added to resuspend the pellet. Supernatants were discarded again and pellets were washed with EtOH/ EtAc (1:1). All samples were vortexed and centrifuged for ten min on 20 000 g. Washing with EtOH/EtAc was repeated three times. Pellet was resuspended in guanidine hydrochloride, vortexed and centrifuged for 10 min on 6 000 g.

**Midgut**

In tubes containing midgut was added PBS (pH 7). Samples were sonicated and centrifuged 10 min on 6000 g. From the supernatant was taken 3 mg of tissue and put in new tubes. PBS was added to make overall volume 500 µl. Streptomycin sulfate was added and protocol was followed as for hemolymph.

**Body wall muscles**

After removing other internal organs and adipose tissue, body wall muscles were crushed in liquid nitrogen, weight was noted and PBS was added. Protocol was followed same as for midgut. Experiments were performed on 7 days old males 24 hours after treatments.

### 2.7.2. GST activity in midgut and body wall muscles

GST activity was measured using the Glutathione S-transferase (GST) Assay Kit (Sigma Aldrich, CS0410). The enzymes protect cells against toxicants by conjugating the thiol group of the glutathione to electrophilic xenobiotics and thereby defend cells against the mutagenic, carcinogenic and toxic effects of the compounds. It utilizes 1-chloro-2,4-dinitrobenzene (CDNB) which is suitable for the broadest range of GST isozymes. Upon conjugation of the thiol group of glutathione to the CDNB substrate there is an increase in the absorbance at 340 nm so all samples were measured at 340 nm on 30°C for 10 min (1 min interval). The experiments were performed 24 hours after treatments.

**Midgut**

Midguts were extracted and weight was noted. PBS was added in all samples – weight of the midgut multiplied by 3. Samples were sonicated and centrifuged 10 min on 10 000 g. Supernatant was used. Master mix was prepared with appropriate amounts of Dulbecco's Phosphate buffer saline, 200 mM L-glutathione reduced and CDNB. Controls were prepared with GST controls and sample buffer from the kit and PBS. UV microplate was used and all samples were double plated.

Standards for protein quantification were serially diluted with PBS. Master mix was prepared with BCA and copper(II)sulfate. All samples and standards were added on microplate and put in thermoshaker for 30 min on 37°C and then read on 562 nm.

**Body wall muscles**

Body wall muscles were crushed in liquid nitrogen as previously described. Protocol for GST was followed as in midgut.

### 2.7.3. Catalase activity in midgut

For measuring catalase activity in midgut, The Amplex Red Catalase Kit (A22180) was used. It was measured spectrophotometrically at 570 nm. *P. apterus* males were dissected and midgut was removed. An appropriate amount of the catalase solution was diluted with reaction buffer for catalase standard curve. Catalase – containing samples were diluted in reaction buffer and used for each reaction. Diluted experimental samples, standard curve samples and control were pipetted in separate well of 96-well microplate. The 40 μM $H_2O_2$ solution was pipetted to each microplate well. Reaction was incubated 30 min at room temperature and then master mix was added to each plate (Amplex Red/HRP working solution) following by incubation in dark at 37°C for 30 min. Absorbance was measured at 570 nm.

## 2.8. Statistical analysis

Statistics were calculated using the graphic software Prism (Graph Pad Software, version 6.01 San Diego, CA, USA). Statistical differences were evaluated by one – way ANOVA with Dunnett's post test (Figs. 3, 4 and 5) and Bonfferoni post test (Figs. 7 and 8). T – test was used for data presented in figures 9 and 10. Experiments were repeated three times and all samples were run in triplicates.

# 3. RESULTS

All experiments were performed on 7 days old *P. apterus* males 4 and/or 24 hours after treatments and run in triplicates.

## 3.1. Protein carbonyls

Protein carbonyl content was measured 4 and 24 hours after treatments with Ringer solution, venom, AKH and AKH + venom co-treatment.

### 3.1.1. Hemolymph

For experiments with hemolymph 1 μl was extracted from each insect. In every treated group were six insects – 24 insects all together. Treatments were repeated three times and graphs present cumulative data collected after measurements. Results are presented in Figure 3. and Figure 4.

There was significant decrease in protein carbonyl content after venom injection compared to control ($p < 0,05$). Protein carbonyl content decreased from 5,2 nmol/mg protein (control) to 3,1 nmol/mg protein (venom). After AKH application, increase in protein carbonyl content to 7,2 nmol/mg protein was recorded but not significant. After venom and AKH co-treatment, protein carbonyl content stayed in range of control level (5,2 nmol/mg protein (Fig. 3).

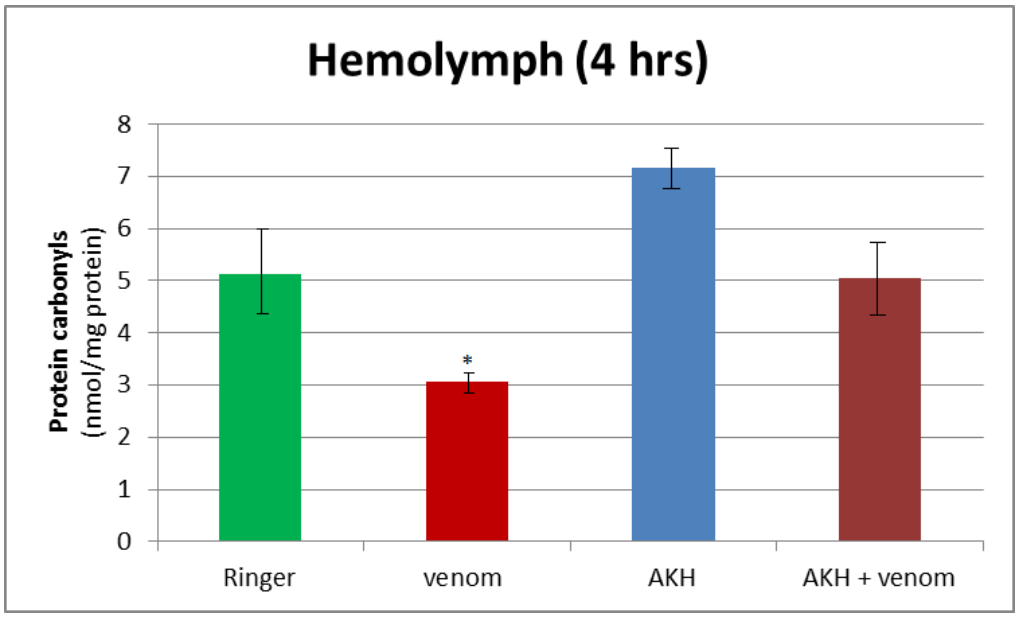


Figure 3. Protein carbonyl content in hemolymph *P. apterus* males after 4 hours treatments with Ringer, venom, AKH and AKH + venom. Presented results are average values ± SD. Statistically significant difference is in group with venom application compared to control (* $p < 0,05$).

Experiments performed 24 hours after treatments showed no significant difference between treated groups and also overall decrease in protein carbonyl content was recorded (Fig 4). Graph presents cumulative data collected after repeating experiment 3 times.

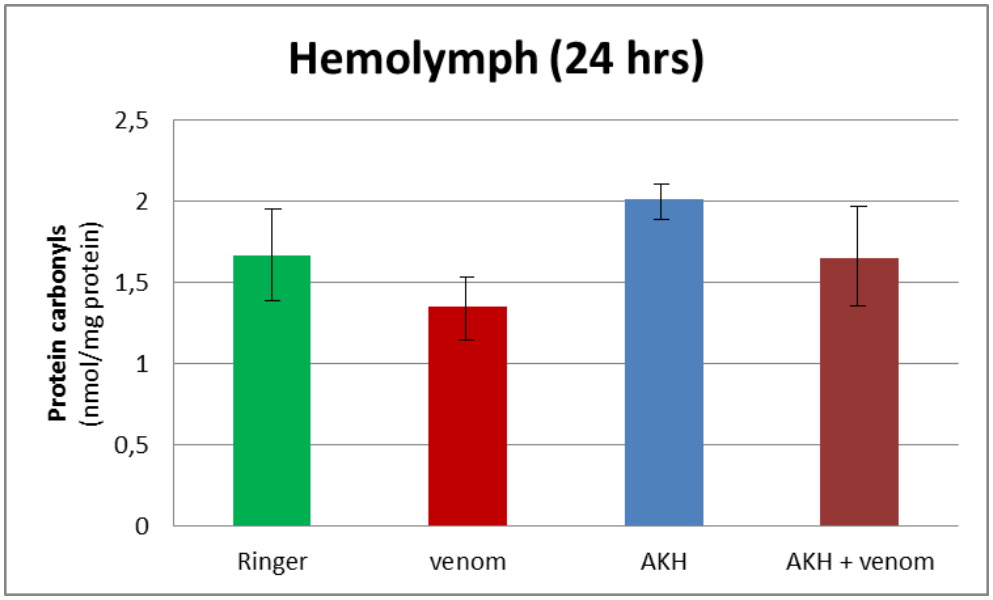


Figure 4. Protein carbonyl content in hemolymph *P. apterus* males after 24 hours treatments with Ringer, venom, AKH and AKH + venom. Presented results are average values ± SD.

### 3.1.2. Midgut

After dissection, one midgut was stored in each tube. In every experimental group were five tubes (five midguts). Experiments were repeated 3 times and graphs present cumulative data collected after analysis. In controls protein carbonyl content was 4,9 nmol/mg protein, and in venom injected groups carbonyl content was 3,86 nmol/mg protein. There was non-significant decrease in protein carbonyl content when venom was injected compared to control. There was no change when AKH was applied, carbonyl content was 4,88 nmol/mg protein which was in range of control content. There was significant increase in protein carbonyl content to 6,95 nmol/mg protein when venom was injected together with AKH application compared to venom only treatment where carbonyl content was 3,86 ($p < 0,05$), (Fig. 5)

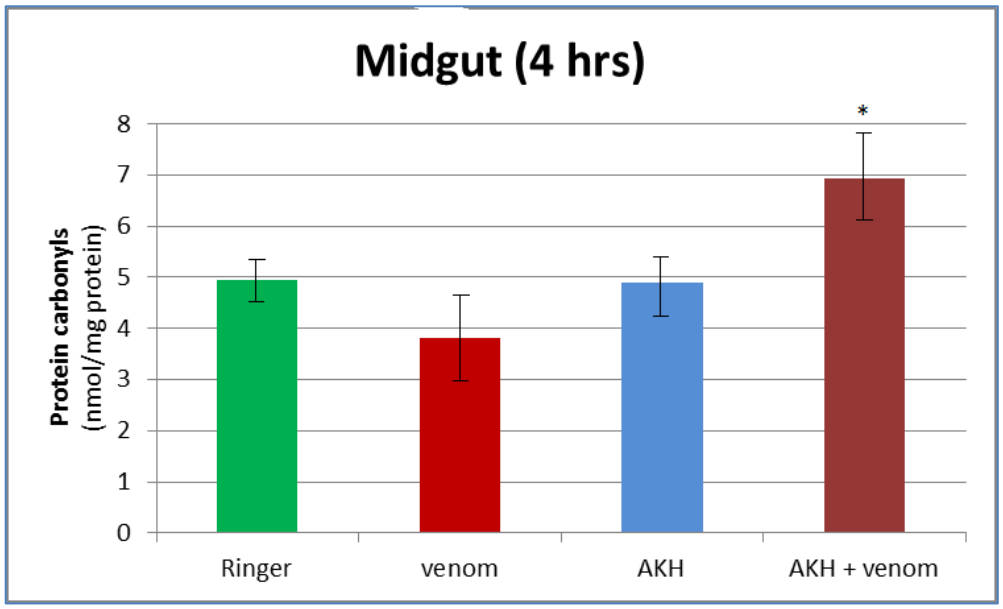


Figure 5. Protein carbonyl content in midgut *P. apterus* males after 4 hours treatments with Ringer, venom, AKH and AKH + venom. Presented results are average values ± SD. Statistically significant difference is in group with AKH + venom application compared to venom only (* $p < 0,05$).

Again, after 24 hours there was decrease in overall content of protein carbonyls where in control content was 2,74 nmol/mg protein (Fig. 6). There was non-significant increase of protein carbonyls after venom injection to 3,63 nmol/mg protein which is inconsistent with previous results presented for midgut 4 hours after treatments. Application of AKH decreased content of protein carbonyls to 1,96 nmol/mg protein compared to control (3,63 nmol/mg protein) and co-application with venom increased the content to 4,52 nmol/mg protein but non-significant.

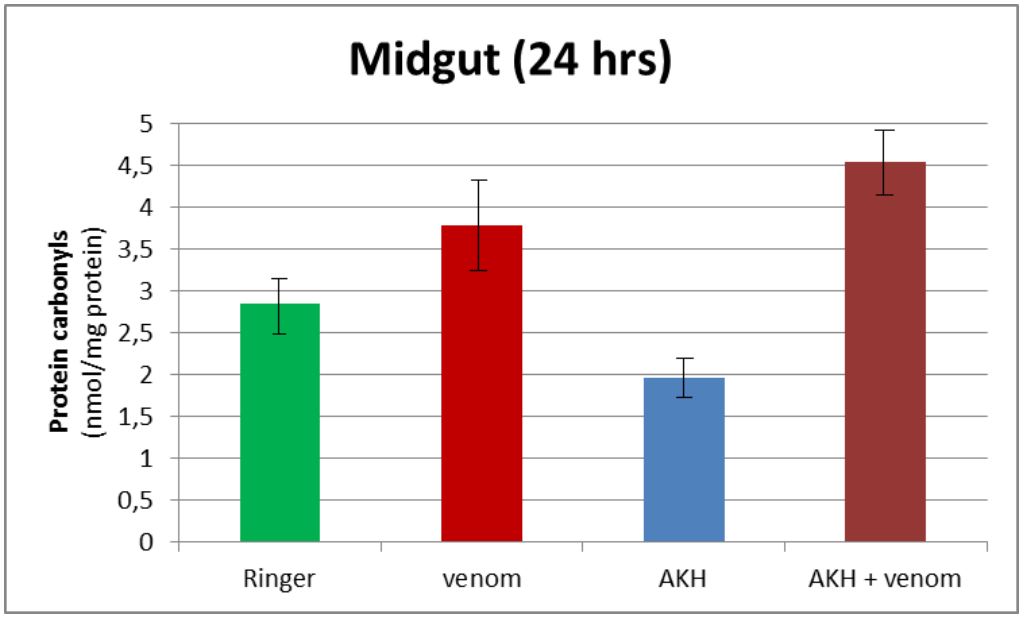


Figure 6. Protein carbonyl content in midgut *P. apterus* males after 24 hours treatments with Ringer, venom, AKH and AKH + venom. Presented results are average values ± SD.

### 3.2.Glutathione S-transferase activity

Glutathione S-transferase activity (GST) was measured 24 hours after treatments with Ringer solution (controls), venom, AKH and AKH + venom.

#### 3.2.1. Midgut

GST activity in *Pyrrhocoris* midgut was the same in Ringer and AKH treated groups and it was 4,47 nmol/µg protein/min. There was significant increase in activity when insects were injected with venom where activity was measured to be 5,66 nmol/µg protein/min. When they were co-treated with venom and AKH activity increased significantly to 5,85 nmol/µg protein/min compared to control and AKH application only ($p < 0,05$, Fig. 7).

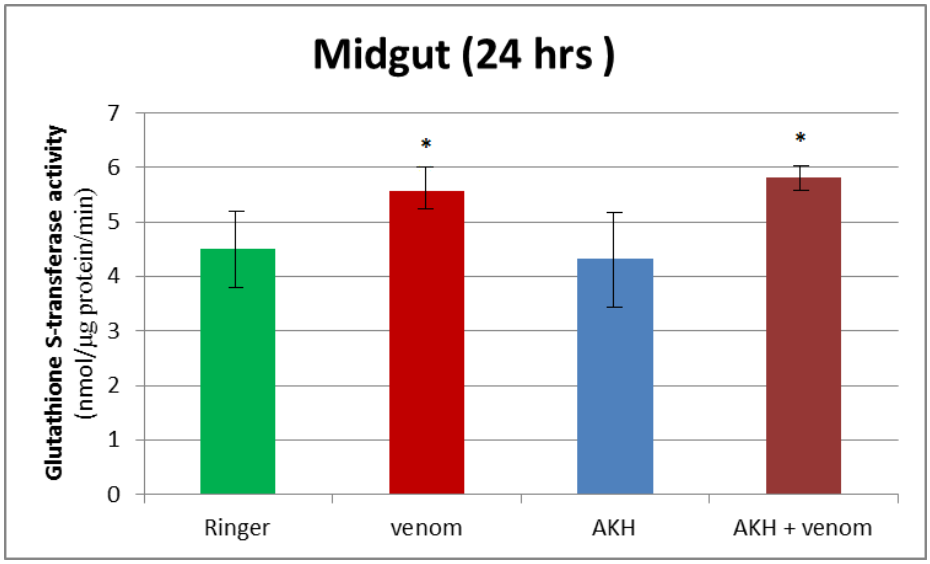


Figure 7. Glutathione S-transferase activity in midgut *P. apterus* males after 24 hours treatments with Ringer, venom, AKH and AKH + venom. Presented results are average values ± SD. Statistically significant difference is in groups treated with venom and AKH + venom compared to control (* $p < 0,05$).

### 3.2.2. Body wall muscles

When GST activity was measured in body wall muscles, there was no significant differences between any of the groups (Fig. 8). All values were in control range.

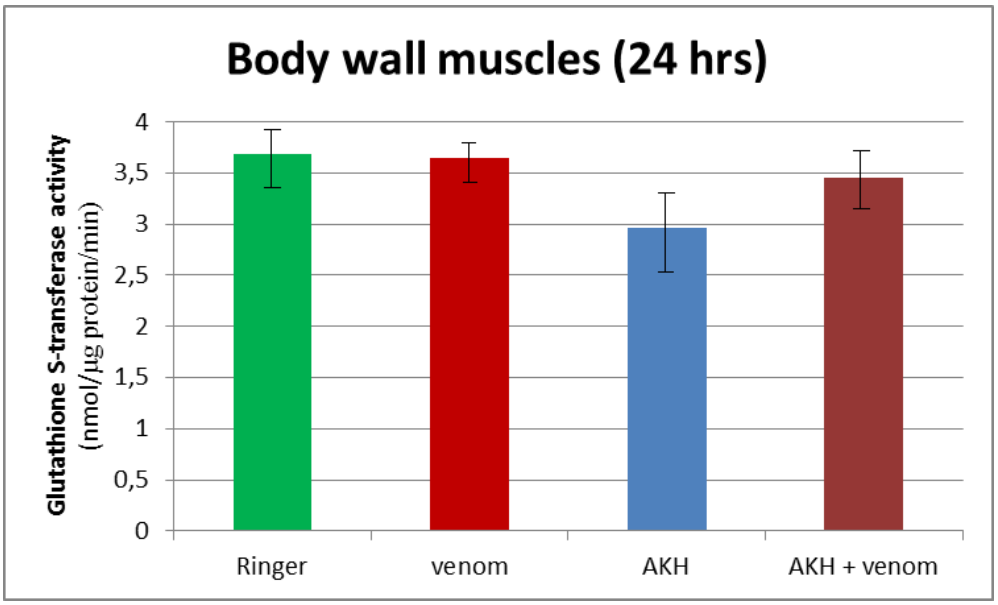


Figure 8. Glutathione S-transferase activity in body wall muscles *P. apterus* males after 24 hours treatments with Ringer, venom, AKH and AKH + venom. Presented results are average values ± SD.

### 3.3. Catalase activity

Catalase activity was measured 24 hours after treatments with Ringer solution (controls), venom, AKH and AKH + venom.

#### 3.3.1. Midgut

There was significant decrease in CAT activity when venom was injected ($p < 0,05$). CAT activity was 1,5 mU/µg protein after venom injection compared to control where activity was 1,98 mU/µg protein. When AKH was applied there was decrease in activity to 1,66 mU/µg protein compared to control. Co – treatment AKH + venom showed significant decrease in CAT activity to 1,51 mU/µg protein compared to control ($p < 0,05$) (Fig. 9).

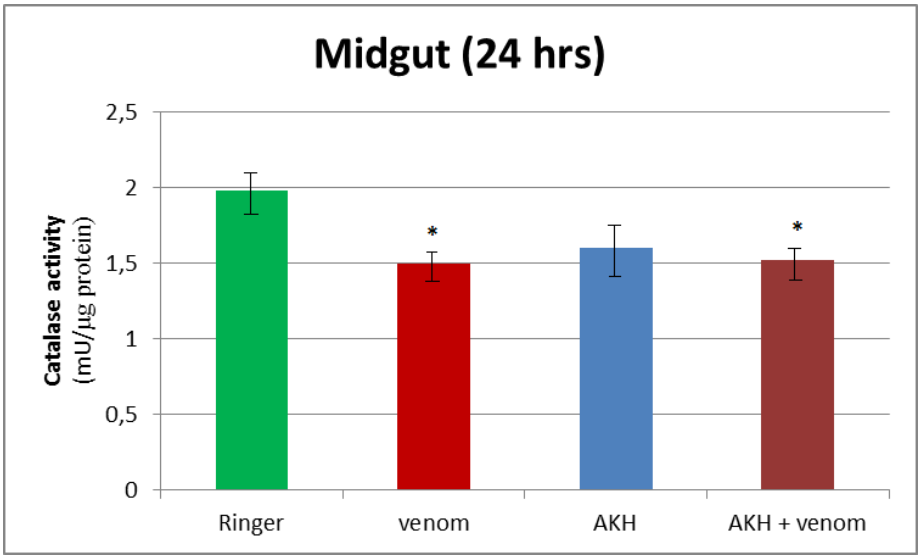


Figure 9. Catalase activity in midgut *P. apterus* males after 24 hours treatments with Ringer, venom, AKH and AKH + venom. Presented results are average values ± SD. Statistically significant difference is in groups treated with venom and AKH + venom compared to control (* $p < 0,05$).

### 3.4. AKH level in CNS

AKH level was measured 24 hours after treatment with venom. On Fig. 10 is visible significant increase of AKH level after venom injection to 1,32 pmol compared to controls where measured AKH level was 0,62 pmol ($p > 0,05$).

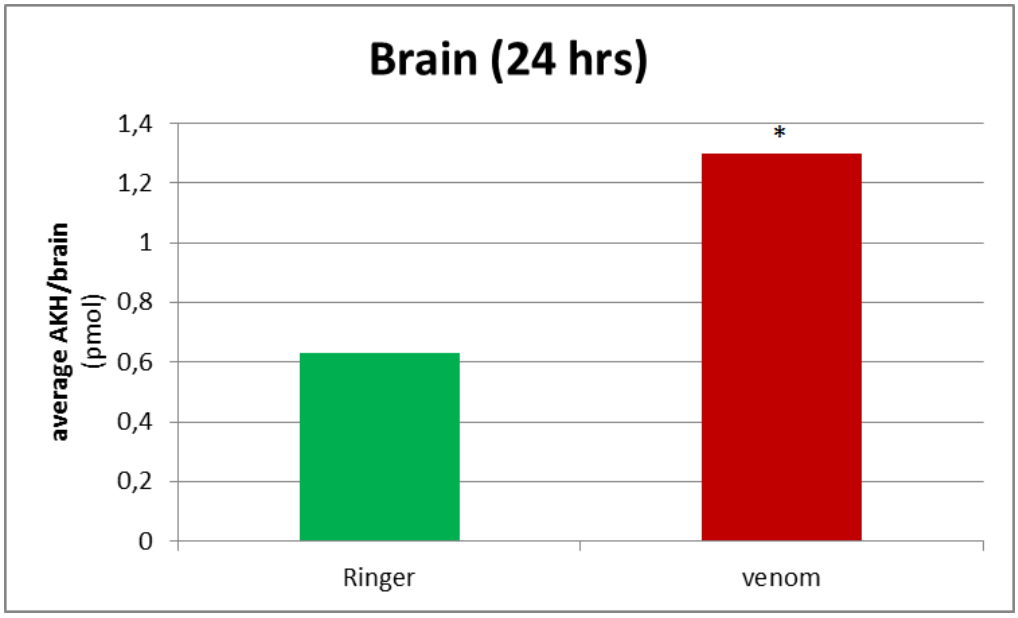


Figure 10. AKH level in central nervous system (CNS) of *P. apterus* males after 24 hours treatment with venom. (* $p < 0,05$)

All insects treated with venom were paralyzed and those with AKH + venom co-treatment were semi-paralyzed.

# 4. DISCUSSION

Adipokinetic hormones are main stress hormones in insects. Gland responsible for production of these hormones is corpora cardiaca – neuroendocrine gland connected with the brain. Their main role is in controlling insect energy metabolism but they have many functions in addition to their metabolic role. AKHs also play important role in insect anti-oxidative stress response (Krishnan *et al.*, 2012). To explore this role in the firebug *Pyrrhocoris apterus*, venom of parasitic wasp *Habrobracon hebetor* was used. Venom blocks receptors on the presynaptic membranes of neuromuscular junctions and inhibits exocytosis in presynaptic vesicles (Pennacchio and Strand, 2006) and thus disabling the transmission of the neuromuscular action potential (Sláma, 2012).

Stress usually increases level of AKH in insect body. Abdul *et al.* in 2017. showed that after injecting venom level of AKH significantly increased in CNS and hemolymph and general metabolism was reduced which was indicated by $CO_2$ production. When venom and AKH were co-applied neuromuscular paralysis was reduced compared to the application of venom only.

In this study, venom of *H. hebetor* was used as stressor and several OS biomarkers were monitored: protein carbonylation, glutathione S-transferase activity and catalase activity.

Protein carbonylation notes the amount of oxidative damage. It can be induced by various ROS or byproducts of oxidative stress. In this research when venom was injected in insects, protein carbonyl level in hemolymph, 4 hours after injection, significantly decreased compared to control. Protein carbonyl content was increased after AKH treatment but its level was as controls after AKH + venom co-treatment (Fig. 3). Usually, chemical stressors such as hydrogen peroxide, paraquat or tannic acid increase protein carbonyl level in insect hemolymph (Bednářová *et al.*, 2013, Kodrík *et al.*, 2007, Večeřa *et al.*, 2007) so this results were unexpected.. After the incubation time was prolonged to 24 hours, same results were obtained, protein carbonyl level declined after venom injection (Fig. 4). It is important to notice that venom obtained from *H. hebetor* is biological stressor and it is possible that decreased carbonylation means that venom has targeted proteins in hemolymph and it induces reduction in carbonyl content of those proteins. Co-treatment with venom and AKH restored carbonyl content on control level after 4 and 24 hours (Fig.3, Fig. 4). As mentioned before, AKH plays important role protecting insect's body

from oxidative damage so if venom causes reduction in carbonyl content in hemolymph then restoring it to control level was expected. Nevertheless, further research is necessary.

When measuring protein carbonyl content in midgut 4 hours after treatments, the data obtained was not as expected and had too many variables after each measuring (Fig. 5) so the time of incubation after treatments was prolonged on 24 hours and data was stabilized (Fig. 6). Even though statistical analysis showed no significant difference, protein carbonyl level slightly increased after venom injection, decreased after AKH application only and both were expected. Večeřa *et al.* (2011) fed tannic acid to *Spodoptera literalis* and noted that midgut protein carbonyl content significantly increased. Similar results were obtained by Kodrík *et al.* (2007) – their experiments were performed on *Leptinotarsa decemlineata* fed on potatoes containing oxidative stress elicitors and protein carbonyl content was also significantly increased in gut tissue. However, increased content of protein carbonyl above control level after co-treatment with venom and AKH was not expected especially when it showed reduced neuromuscular paralysis compared to the application of venom only (observation) but also demonstrated by Abdul *et al.* (2017).

Results were not positive (there was no significant differences between treated groups), but inducing carbonylation of proteins can be rather difficult. It is possible that prolonged time to 24 hours was not long enough to increase protein carbonyl level in midgut of *P. apterus.*

Negative results were obtained when protein carbonyl content was measured in body wall muscles (data not shown). It suggests that carbonylation does not affect that part of insect's body but further research is necessary.

Another OS biomarker was measured – glutathione S-transferase. GSTs are large group of detoxifying enzymes. Their substrate specifity is extensive and includes compounds like carbonyls, quinones and organic hydroperoxides (Manerwick *et al.*, 1988; Sawicki *et al.*, 2003). Conjugation of GSH to electrophilic centers of substrates via sulfhydryl group is catalysed by GSTs.

Injection of venom in *P. apterus* body resulted in increased activity of GST in midgut 24 hours after treatment (Fig. 7) which was expected. Generally, increased activity of this enzyme is linked to major classes of insecticides (Prapanthadara *et al*., 1993; Huang *et al*., 1998; Vontas *et al*., 2001). Interestingly, co-application of venom and AKH did not result

in lowering of GST activity. As stated before, AKH has significant antioxidative role in insects body so same activity level in both groups was not expected.

Experiments with body wall muscles showed no significant difference in GST activity between treated groups thus following protein carbonyl negative trend (Fig. 8).

Catalase activity was measured in midgut 24 hours after treatments. Significant reduction in activity was documented in groups treated with venom and co-treated with venom and AKH compared to control (Fig. 9). Application of AKH alone did not increase CAT activity above control level. Co-treatment with venom and AKH does not restore catalase activity to control level. Results indicate possibility of different factors that might be involved and that they cooperate with AKH action. *In vitro* experiments were performed on CNS of *P. apterus* to study AKH role in OS elicited by application of hydrogen peroxide (Bednarova *et al.,* 2013). CNS was incubated with hydrogen peroxide and CAT activity was reduced and co-treatment with AKH did not restore catalase activity to control level. In another paper was shown how expression of *CAT* genes were enhanced during OS elicited by tannic acid in *Spodoptera littoralis* larvae and then reduced to control level when feeding with tannic acid was followed by AKH treatment which indicated role of AKH in the control mechanism. However, activity of CAT was not stimulated by tannic acid feeding which was expected after *CAT* gene expression (Večeřa *et al.,* 2011).

It is possible that *CAT* gene of *P. apterus* has reduced expression upon venom application and thus reduced activity of CAT. AKH activity might be stressor and/or species depended but further research is necessary.

AKH level was significantly increased when measured in brain of firebug 24 hours after venom application (Fig. 10). This result was expected as Abdul *et al.* (2017). recently reported that expression of *Akh* genes was significantly increased in CNS of *P. apterus* after venom application. AKH has primary role in insect anti-stress defense mechanism and functionally is like mammalian glucagon (Alquicer *et al*., 2009; Bednářová *et al*., 2013). Beside its anti-stress role, it is involved in other actions as cardiostimulation, inhibition of RNA synthesis, proteins and fatty acids in fat body, induction of gene expression for a cytochrome P-450 enzyme and stimulation of oxidation of substrates by the flight muscles (Gäde, 2004; Gäde *et al*., 1997).

# 5. CONCLUSIONS

When co-treated with venom and Pyrap-AKH, protein carbonyls returned to control level in hemolymph but increased above control level in midgut. Venom elicited activity of GST in midgut but decreased CAT activity. After co-treatment GST and CAT activity did not change compared to venom treatment which was not expected. Quantification of Pyrap-AKH showed elicited AKH level in CNS after venom injection.

Body wall muscles were not affected by venom.

Study demonstrated that the co-application of *H. hebetor* venom with Pyrap-AKH into *P. apterus* body significantly reduced total neuromuscular paralysis compared to the application of venom only (observation).

It is apparent that venom elicited oxidative stress in *P. apterus* but co-application of venom and AKH did not match the expectation. Further study is necessary to fully understand AKH response but also venom targets in host's body.

The study is academically interesting and further research might contribute data that could be applicable in human or veterinary medicine in the future.

# 6. LITERATURE


Abdul, H., Mishra, A., Kodrík, D. (2017) Beneficial effect of adipokinetic hormone on neuromuscular paralysis in insect body elicited by braconid wasp venom. Comparative Biochemistry and Physiology 196: 11–18.

Alquicer, G., Kodrík, D., Krishnan, N., Večeřa, J., Socha, R. (2009) Activation of insect anti-oxidative mechanisms by mammalian glucagon. Comparative Biochemistry and Physiology 152: 226–233.

Altuntaş, H., Kiliç, A. Y. and Zeytİnoğlu, H. S. (2010) The effects of parasitism by the ectoparasitoid *Bracon hebetor* Say (Hymenoptera: Braconidae) on host hemolymph proteins in the Mediterranean flour moth *Ephestia kuehniella* Zeller (Lepidoptera: Pyralidae). The Turkish Journal of Zoology 34: 409–416.

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

Bednářová, A., Kodrík, D., Krishnan, N. (2013) Unique roles of glucagon and glucagon-like peptides: parallels in understanding the functions of adipokinetic hormones in stress responses in insects. Comparative Biochemistry and Physiology 164: 91–100.

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

Benson, J. F. (1974) Population dynamics of *Bracon hebetor* Say (Hymenoptera: Braconidae) and *Ephestia cautella* Walker (Lepidoptera: Phycitidae) in a laboratory ecosystem. Journal of Animal Ecology 43: 71-84.

Bolognesi, R., Beermann, A., Farzana, L., Wittkopp, N., Lutz, R., Balavoine, G., Brown, S. J., Schroder, R. (2008) *Tribolium* Wnts: evidence for a larger repertoire in insects with overlapping expression patterns that suggest multiple redundant functions in embryogenesis. Development Genes and Evolution 218: 193 – 202.

Boyland, E. and Chasseaud, L. F. (1969) The role of glutathione and glutathione S-transferases in mercapturic acid biosynthesis. Advances in Enzymology 32: 173-219.

Butterfield, D. A., Stadtman, E. R. (1997) Protein oxidation processes in aging brain. Advances in Cell Aging and Gerontology 2: 161–191.

Chapman, R. F. (1969) The Insects, Endocrine system. 5th ed., New York, Cambridge University Press

Che-Mendoza, A., Penilla, R., Rodríguez, D. (2009) Insecticide resistance and glutathione S-transferases in mosquitoes: A review. African Journal of Biotechnology 8: 1386–1397.

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

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

Gäde, G., (2004) Regulation of intermediary metabolism and water balance of insects by neuropeptides. Annual Review of Entomology 49: 93–113.

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

Grimsrud, P. A., Xie H., Griffin T. J., Bernlohr D. A. (2008) Review Oxidative stress and covalent modification of protein with bioactive aldehydes. The Journal of Biological Chemistry: 32-41.

Haddad, J. J. (2002) Science review: Redox and oxygen-sensitive transcription factors in the regulation of oxidant-mediated lung injury: role for nuclear factor-κB. Critical Care 6: 481–490.

Ho, E., Karimi Galougahi, K., Liu, C. C., Bhindi, R., Figtree, G. A. (2013) Biological markers of oxidative stress: Applications to cardiovascular research and practice. Redox Biology 1: 483–491.

Hodek, I. (1968) Diapause in females of *Pyrrhocoris apterus* Heteroptera. Acta Entomologica Bohemoslovaca 65: 422–435.

Hodkova, M., Hodek, I. (2004) Photoperiod, diapause and cold-hardiness. European Journal of Entomology 101: 445–458.

Honěk, A. (1976) Factors influencing the wing polymorphism in *Pyrrhocoris apterus* Heteroptera, Pyrrhocoridae. Zoologische Jahrbucher 103: 1–22.

Honěk, A. (1981) Temperature and wing polymorphism in natural populations of *Pyrrhocoris apterus* L. Heteroptera, Pyrrhocoridae. Zoologische Jahrbucher 108: 487–501.

Honěk, A. (1995) Factors and consequences of a non-functional alary polymorphism in *Pyrrhocoris apterus* Heteroptera: Pyrrhocoridae. Researches in Population Ecology 37: 111–118.

Huang, H. S., Hu, N. T., Yao, Y. E., Wu, C. Y., Chiang, S. W. and Sun, C. N. (1998) Molecular cloning and heterologous expression of a glutathione S-transferase involved in insecticide resistance from the diamondback moth, *Plutella xylostella*. Insect Biochemistry and Molecular Biology 28: 651–658.

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

Kodrík D., Marco H. G., Simek P., Socha R., Stys P., Gäde G. (2010) The adipokinetic hormones of Heteroptera: A comparative study. Physiological Entomology 35: 117–127.

Kodrík D., Šimek P., Lepša L., Socha R. (2002) Identification of the cockroach neuropeptide Peram-CAH-II as a second adipokinetic hormone in the firebug *Pyrrhocoris apterus*. Peptides 23: 583–585.

Kodrík D., Šimek P., Lepša L., Socha R. (2002b) Identification of the cockroach neuropeptide Peram-CAH-II as a second adipokinetic hormone in the firebug *Pyrrhocoris apterus*. Peptides 23: 583–585.

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

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 Biochemistry and Molecular Biology 30: 489–498.

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

Kodrik, D., Staškova, T., Jedličkova, V., Weyda, F., Zavodska, R., Pflegerova, J. (2015) Molecular characterization, tissue distribution and ultrastructural localization of adipokinetic hormones in the CNS of the firebug *Pyrrhocoris apterus* (Heteroptera, Insecta). General and Comparative Endocrinology 210: 1–11.

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.

Kovalenkov, V. G. (1984) The biomethod in integrated protection of cotton. Zashchita rastenii Journal 8: 12–14.

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

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

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

Levine, R. L. (1988) Review: Carbonyl modified proteins in cellular regulation, aging, and disease. Free Radical Biology and Medicine 32: 790-796.

Lowenstein C. J., Dinerman J. L., Snyder S. H. (1994) Nitric-oxide - a physiological messenger. Annals of Internal Medicine 120: 227–237.

Lukas, J. and Slama, K. (2011) Myogenic nature of insect heartbeat and intestinal peristalsis, revealed by neuromuscular paralysis caused by the sting of a braconid wasp. Journal of Insect Physiology 57: 251–259.

Mannervik, B., Danielson, U. H., Ketterer, B. (1988) Glutathione transferases - Structure and catalytic activity. Critical Reviews in Biochemistry and Molecular Biology 23: 283–337.

Moreau, S. J. and Guillot, S. (2005) Advances and prospects on biosynthesis, structures and functions of venom proteins from parasitic wasps. Insect Biochemistry and Molecular Biology 35: 1209-1223.

Morgan, T. H. (1911) An attempt to analyze the constitution of the chromosomes on the basis of sex – limited inheritance in Drosophila. Journal of Experimental Zoology 11: 365 – 413.

Nay, J. E., Perring, T. M. (2005) Impact of ant predation and heat on carob moth (Lepidoptera: Pyralidae) mortality in California date gardens. Journal of Economic Entomology 98: 725–731.

Pennacchio, F., Strand, M. R. (2006) Evolution of developmental strategies in parasitic Hymenoptera. Annual Reviews in Entomology 51: 233–258.

Pennacchio, F., Strand,M. R. (2006) Evolution of developmental strategies in parasitic Hymenoptera. Annual Reviews in Entomology 51: 233–258.

Prapanthadara, L., Hemingway, J. and Ketterman, A. J. (1993) Partial purification and characterization of glutathione S- transferase involved in DDT resistance from the mosquito *Anopheles gambiae*. Pesticide Biochemistry and Physiology 47: 119–133.

Quistad, G. B., Nguyen, Q., Bernasconi, P., Leisy, D. J. (1994) Purification and characterization of insecticidal toxins from venom glands of the parasitic wasp, *Bracon hebetor*. Insect Biochemistry and Molecular Biology 24: 955–961.

Reiter R. J., Melchiorri D., Sewerynek E. (1995) A review of the evidence supporting melatonin's role as an antioxidant. Journal of Pineal Research 18: 1–11.

Requena J. R., Levine R. L., Stadtman E. R. (2003) Recent advances in the analysis of oxidized proteins. Amino Acids 25: 221–226.

Sawicki, R., Singh, S.P., Mondal, A. K., Benes, H., Zimniak, P. (2003) Cloning, expression and biochemical characterization of one ε-class (GST-3) and ten Δ-class (GST-1) glutathione S-transferases from *Drosophila melanogaster*, and identification of additional nine members of the Epsilon class. Biochemical Journal 370: 661–669.

Sayre, L. M., Lin, D., Yuan, Q., Zhu, X., Tang, X. (2006) Review Protein adducts generated from products of lipid oxidation: focus on HNE and one. Drug Metabolism Reviews 38: 651-675.

Sláma, K. (2012) Neuromuscular Paralysis Induced in Insect Larvae by the Proteinic Venom of a Parasitic Wasp. In: Berhardt, L. V. (Ed.), Paralysis: Causes, Classification and Treatments, Vol. 43. Advances in Medicine and Biology. Nova Science Publishers, New York, 195–212.

Socha R., Kodrík D., Zemek R. (1999b) Adipokinetic hormone stimulates insect locomotor activity. Naturwissenschaften 27: 85–86.

Socha R., Kodrík D. (1999a) Differences in adipokinetic response of *Pyrrhocoris apterus* Heteroptera in relation to wing dimorphism and diapause. Physiological Entomology 24: 278–284.

Socha R., Šula J., Zemek R. (1998) Feeding behaviour, digestive physiology and lipid content in macropterous females of *Pyrrhocoris apterus* L. Heteroptera: Pyrrhocoridae. Physiological Entomology 23: 1–96.

Socha R., Zemek R. (2000) Locomotor activity in adult *Pyrrhocoris apterus* Heteroptera in relation to sex, physiological status and wing dimorphism. Physiological Entomology 25: 383–389.

Stadtman E. R., Levine R. L. (2000) Review Protein oxidation. Ann NY Acad Sci. 899:191-208.
Story, P. (2005) Reactive oxygen species in tumor progression, Frontiers in Bioscience 10: 1881–1896.

Šula J., Socha R., Zemek R. (1998) Wing morph-related physiological differences in adults of temperate population of *Pyrrhocoris apterus* L. Heteroptera: Pyrrhocoridae. Comparative Biochemistry and Physiology 121: 365–373.

Večeřa J., Krishnan N., Mithöfer A., Vogel H., Kodrík D. (2011) Adipokinetic hormone-induced antioxidant response in *Spodoptera littoralis.* Comparative Biochemistry and Physiology 155: 389–395.

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

Visser, B. J., Labruyere, W. T., Spanjer,W., Piek, T. (1983) Characterization of two paralysing protein toxin (A-MTX and B-MTX) isolated from a homogenate of the wasp *Microbracon hebetor* (Say). Comparative Biochemistry and Physiology 7: 523–530.

Vontas, J. G., Small, G. J. and Hemingway, J. (2001) Glutathione S- transferases as antioxidant defence agents confer pyrethroid resistance in *Nilaparvata lugens*. Biochemical Journal 357: 65–72.

Zhang, R., Brennan, M. L., Fu, X., Aviles, R. J., Pearce G. L., Penn M. S., Topol E. J., Sprecher D. L., Hazen S. L. (2001) Association between myeloperoxidase levels and risk of coronary artery disease. The Journal of the American Medical Association 286: 2136–2142.

Web 1. https://www.britannica.com/animal/insect/Nervous-system (12.10.2018.)