University of South Bohemia

Faculty of Science

EFFECT OF ADIPOKINETIC HORMONE ON ACTIVITY OF DIGESTIVE ENZYMES IN *Drosophila melanogaster*

Bachelor thesis

Michaela Beňová

Biological Chemistry

Supervisor: Prof. RNDr. Dalibor Kodrík, CSc.

České Budějovice 2017

Beňová, M., 2017: Effect of adipokinetic hormone on activity of digestive enzymes in *Drosophila melanogaster*, 51 p., Bc. thesis in English, Faculty of Science, University of South Bohemia, České Budějovice, Czech Republic.

Annotation

The aim of this thesis was to determine the role of adipokinetic hormone and adenosine in stimulation of enzyme functions in the gut of the fruit fly *Drosophila melanogaster* using AKH-def, AKHR-def, AdoR-def and double AKH-def-AdoR-def mutants.

Declaration

I hereby declare that I have worked on my bachelor thesis independently and used only the sources listed in the bibliography.

I hereby declare that, in accordance with Article 47b of Act No. 111/1998 in the valid wording, I agree with the publication of my bachelor thesis, in full to be kept in the Faculty of Science archive, in electronic form in publicly accessible part of the STAG database operated by the University of South Bohemia in České Budějovice accessible through its web pages. Further, I agree to the electronic publication of the comments of my supervisor and thesis opponents and the record of the proceedings and results of the thesis defence in accordance with aforementioned Act No. 111/1998. I also agree to the comparison of the text of my thesis with the Theses.cz thesis database operated by the National Registry of University Theses and a plagiarism detection system.

České Budějovice, 13.8.2017 ……...

Michaela Beňová

Acknowledgement

First, I would like to thank my supervisor prof. RNDr. Dalibor Kodrík CSc., for the opportunity to work in his laboratory, his patience and help during the practical as well as the theoretical part of the thesis. Many thanks also go to all the people from the laboratory, for their optimistic support and for providing the working material. I would also like to thank my friends and classmates for their support and many wonderful moments I experienced with them. Last but not least I thank to my family, for their never ending support, love, and interest in my studies and everything I have ever done.

The study was supported by the project No. 17-03253S from the Czech Science Foundation (DK).

Part of the results from this bachelor thesis (section 4.1) is involved into the manuscript that has been recently submitted into the *Physiological Entomology* journal; the article has not been published yet. The manuscript of the mentioned article can be found in the Appendix (section 8.).

CONTENT

1. INTRODUCTION

1.1 Insect digestive system

Food intake and efficient digestion of food into nutrients is necessary for the maintenance of homeostasis and fulfilling physiological tasks in all animal organisms. Animals had to transit from an intracellular to an extracellular mode of digestion during their evolution. This led to the emergence of the gastrointestinal tract within the body cavity. In Drosophila and other bilaterians, the digestive tract further evolved into a succession of histologically distinct regions accommodated to specific digestive needs. In other words, because of the dietary requirements of Drosophila (usually fermenting fruit) an alimentary canal developed (or the gut). The gut of Drosophila is made of a simple epithelium, which is surrounded by visceral muscles, nerves, and tracheae. The digestive system is divided into three main structures: the foregut (stomodeum), the midgut (mesenteron), and the hindgut (proctodeum) with midgut being further divided into six parts (Figure 1). The foregut and hindgut are of ectodermal origin and therefore lined with a cuticle. (Lemaitre *et al.,* 2013). The foregut provides for ingestion, storage, grinding and transport of food to the midgut. In the hindgut, water, salts and other useful molecules are absorbed from remaining unabsorbed food prior to excretion of the faeces by anus. The midgut epithelium is of endodermal origin and instead of cuticular lining it possess a peritrophic membrane, consisting of a network of chitin fibrils in a protein-glycoprotein matrix. Peritrophic membrane separates the midgut from food bolus and also serves as a sieve allowing passage of small molecules while large molecules, bacteria and food particles are restricted from passing. Together with the caeca (in some literature considered a part of midgut), the midgut is the major site of digestive enzymes production and secretion. As a result, it is the main site for digestion of food and absorption of nutrients (Gullan, 2005) Physicochemical conditions in the gut (pH) or enzymatic activity of microbes also affect the digestion. The pH in the midgut is usually around 6-7.5. Moreover, gut contractions are crucial for the food mobility and flow of digestive enzymes. The digestive tract is known to be under hormonal control. That is supported by the fact that the insect gut together with mouthparts are innervated by the central and stomatogastric nervous systems (CNS and SNS, respectively) (Spit *et al.,* 2012).

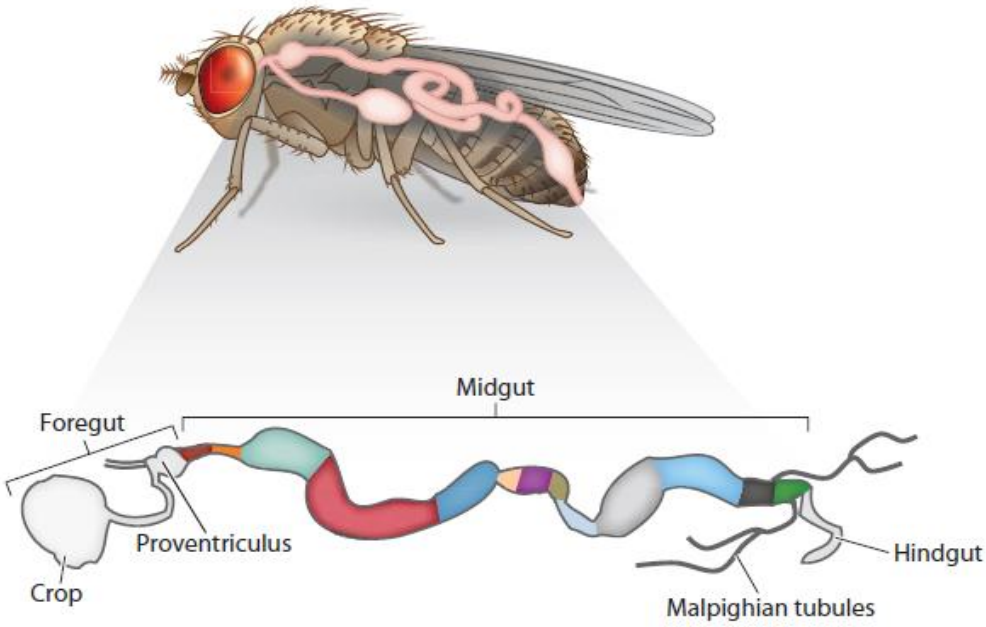

Figure 1: Digestive tract of *Drosophila melanogaster.* After Lemaitre (2013).

1.2 Insect endocrine system

Insect endocrine system is similar to that of vertebrates. It responds to stimuli by releasing hormones, which serve as chemical messengers, into the insect blood stream (haemolypmph stream). Among organs in which the production of hormones is secondary function (e.g. ovaries, testes), several endocrine centres which produce hormones as their primary function are known: neurosecretory cells, corpora cardiaca, prothoracic glands and corpora allata (Gullan, 2005).

Neurosecretory cells (NSC) are modified neurons found through multiple nervous systems (CNS, SNS,...), but they mainly occur in the brain. They produce hormones which act either directly on the organs of insect or on the other endocrine organs, which are then stimulated to produce hormones. It is said that they serve as a link between the nervous system and the endocrine system (Gullan, 2005; Chapman, 1998).

Prothoracic glands are paired glands located in the thorax or the back of the head. They release moulting hormones (ecdysteroids). When most insects reach the adult stage, the prothoracic glands break down and moulting never occurs again (Gullan, 2005).

Corpora cardiaca are mostly a pair of neuroglandular bodies located behind the brain and on either side of the aorta. The most important functions are synthesis, storage and release of adipokinetic

hormones (see chapter 1.3 for details); in some insect species corpora cardiaca release also prothoracicotropic hormone (PTTH), which stimulates secretion of the hormones produced by the prothoracic glands.

Corpora allata are small, paired glandular bodies located on both sides of the foregut, or they can be fused into one organ. They secrete juvenile hormones, which are important in regulating metamorphosis and yolk deposition in eggs (Gullan, 2005; Chapman, 1998).

Organization of the above mentioned insect endocrine system is not uniform. For example in juvenile stadia of higher Diptera (including *D. melanogaster*) the endocrine glands fuse together to create a ring gland (Figure 1) producing all important insect developmental and metabolic hormones.

1.2.1 Hormones

Three groups of hormones are crucial for control of the main biological functions in insects. These are: juvenile hormones (JHs), ecdysteroids and neurohormones (also called neuropeptides).

Juvenile hormones are acyclic sesquiterpenoids that have two major roles – regulation of juvenile development by preventing a premature metamorphosis, and control of reproduction in adults.

Ecdysteroids are important in the eliciting of insect moulting and metamorphosis. They are derived from sterols (e.g. cholesterol) which cannot be synthesized *de novo* by insects. The most common are ecdysone, which is usually converted to more active 20-hydroxyecdysone in target tissues. Ecdysteroids are also produced in adult gonads and are therefore involved in gamete maturation.

Neurohormones are the largest class of insect hormones. Since they are (small) peptides, they are often called neuropeptides. They regulate insect development, metabolism, reproduction, homeostasis, muscle control, behaviour, etc. (Chapman, 1998). Some neuropeptides present in the SNS have been localized in the midgut endocrine cells, suggesting a role in the regulation of digestive processes. In addition, some regulatory neuropeptides show stimulating or inhibitory effects on enzyme activity levels in the gut of insects, indicating that the control of enzyme release in response to food is probably under neuropeptidergic control. (Spit *et al*., 2012).

1.3 Adipokinetic hormones

Adipokinetic hormones (AKH) are short insect neuropeptides produced, stored and released by endogenous neurosecretory cells of corpora cardiaca. They belong to the adipokinetic hormone/red pigment concentrating hormone (AKH/RPCH) family and up to date, about 50 different forms have been identified (Gäde, 2009; Kodrík *et al.*, 2010). AKH hormone was first isolated and sequenced from *Locusta migratoria migratorides*, and called Locmi-AKH-I (Stone *et al.*, 1976). AKHs are involved in the metabolism and the release of energy in insects (Gäde et al., 1997; Kodrík, 2008). Structurally, AKHs consist of eight to ten amino acids, with the amino acids tryptophan and glycine at position 8 and 9 (if present) and at least two aromatic amino acids in the whole peptide (Gäde & Goldsworthy, 2003). In addition, N-terminus is blocked by pyroglutamate residue and C-terminus is blocked by amido group in all AKHs except for an AKH of the butterfly *Vanessa cardui* (Köllisch *et al.*, 2000; Kodrík, 2008). For *D. melanogaster*, the AKH hormone is called Drome-AKH, with the amino acid sequence: pGlu-Leu-Thr-Phe-Ser-Pro-Asp-Trp-NH_2 (Schaffer *et al*., 1990; Noyes *et al*., 1995).

1.3.1 Functions of AKHs

Although the main function is undoubtedly the control of energy metabolism, the hormones are pleiotropic, meaning that they have many other effects on the insect body. In general, they can be considered stress hormones – they stimulate catabolic processes, making energy more available. The actions of AKHs in the insect body can be divided into three groups: general metabolic actions, biochemical actions and physiological actions.

1.3.1.1 General metabolic actions

These actions eventually result in the release of stored fuels for flight. Firstly, AKHs mobilize triacylglycerol (TAG) stored in the body, which results in the release of lipid diacylglycerol (DAG) into the haemolymph. DAG acts as a long-term fuel for flight (Arrese *et al.*, 2001; Van der Horst *et al.*, 1999). Moreover, they mobilize carbohydrate trehalose from glycogen (Keeley *et al.*, 1996; Gäde & Auerswald, 2002a) and increase the level of amino acid proline (Gäde & Auerswald, 2002a).

1.3.1.2 Biochemical actions

On the biochemical level, AKHs activate adenylate cyclase and lipase (Spencer & Candy, 1976), glycogen phosphorylase (Van Marrewijk *et al.*, 1980), phospholipase C (Vroemen *et*

al., 1997) and antioxidant mechanism (Kodrík *et al.*, 2007). Furthermore they stimulate the biosynthesis of mitochondrial cytoheme (Keeley *et al.*, 1991) and increase the lipid-carrying capacity of lipoprotein carriers (Kanost *et al.*, 1990). AKHs also inhibit the syntheses of RNA (Kodrík & Goldsworthy, 1995), proteins (Carlisle & Loughton, 1979) and lipids (Gokuldas *et al.*, 1988).

1.3.1.3 Physiological actions

Among physiological functions, we include stimulation of heart beat (Scarborough *et al.*, 1984) and general locomotion (Socha *et al.*, 1999), inhibition of egg maturation (Lorenz, 2003), and regulation of starvation-induced foraging behaviour (Lee & Park, 2004). These hormones also increase muscle tonus (O´Shea *et al.*, 1984), enhance immune responses (Goldsworthy *et al.*, 2002, 2003a) and affect digestive system (Kodrík *et al.*, 2012).

1.3.2 Mode of action

AKH peptides are not able to penetrate cell membrane freely, therefore the information is transported through a membrane receptor(s) and stimulates the release of particular enzyme. When peptide hormones bind to their receptors in the membrane, a series of signal transduction events is initiated. Those lead to the activation of the targeted enzymes. One of the transduction events is the change of conformation of the receptor after binding of AKH. Such changed receptor interacts with GTP-binding protein (G-protein) which transduces the signal to an enzyme adenylcyclase, which produces the second messenger cAMP. cAMP can either activate the targeted enzyme or get into the nucleus and activate DNA transcription of the targeted enzyme.

1.4 Adenosine

Adenosine is a purine nucleoside composed of adenine (purine derivative) attached to a sugar molecule (ribose) by glycosidic bond. It can be found in all living organisms, in the intracellular and the extracellular matrix of tissues. If attached to a deoxyribose sugar molecule, adenosine is one of the structural units of both, DNA and RNA (nucleic acids). It regulates several processes: oxygen and cellular energy homeostasis, metabolism of fats and glycides and it is also involved in immune and nervous responses (Jacobson & Gao, 2009). Sometimes, adenosine is considered a stress hormone, due to its function as a signalling molecule (Haskó *et* al., 2002). Under normal conditions, the concentration of adenosine in body fluids is 20 – 200 nM, however, under stressful conditions the concentration rises to mM

(Ferdholm, 2010; Kučerová *et al.*, 2012). The concentration is controlled by the enzymes of adenosine degradation – adenosine deaminase and adenosine kinase. Adenosine deaminase provides for the conversion of adenosine into inosine and adenosine kinase catalyses the phosphorylation of adenosine leading to AMP, after it has been transported into cytoplasm by membrane transporters. AMP gets further phosphorylated into ADP by adenylate kinase and ADP is phosphorylated into ATP by creatine kinase (Lloyd & Fredholm, 1995). Enzymes enabling the formation of adenosine are ecto-5´-nucleotidase (increases extracellular adenosine by hydrolysis of extracellular adenine), cytosolic 5´- nucleotidase (dephosphorylates intracellular AMP to form adenosine) and S-adenosyl-L-homocysteine hydrolase (catalyses hydration of S-adenosine-L-homocysteine into adenosine and homocysteine). The transport of adenosine molecules through the cell membranes is mediated by nucleoside transporters. Two families of such transporters are known: concentrative and equilibrative (Rose & Coe, 2008; Molina-Arcas *et al.*, 2009). The former provides for influx against the concentration gradient, making use of the transmembrane sodium gradient. The latter, the equilibrative transporters provide for the both-way transport down the concentration gradient (Baldwin *et al.*, 2004; Gray *et al.*, 2004). The extracellular adenosine reacts with the membrane adenosine receptors (AdoRs). They are a form of G-protein coupled receptors which are involved in many transductional mechanisms. In mammals, they are divided into four groups: A_1, A_{2A}, A_{2B} and A_3. Each group has different tissue distributions, modes of action and G-protein binding preferences. Activation of A_1 and A_3 results in the decrease of cAMP concentration (they couple to the G_I (inhibitory) protein family). On the other hand, an increase of cAMP concentration is observed after the activation of A_{2A} and A_{2B} (coupled to the G_S (stimulating) protein family). Only one AdoR was found in *D. melanogaster.* It couples to the G_S protein family, therefore it stimulates cAMP production (Doleželová *et al.*, 2007; Kučerová *et al.*, 2012).

AKH and adenosine share some actions on cellular and physiological level. Both adenosine and AKH signals are mediated by G-protein coupled receptors, which affect cAMP production (Park *et al.*, 2002; Jacobson & Gao, 2009). Moreover, they both affect the regulation of energy and oxygen metabolism, physiology of adipose tissue and immune and neural responses (Kodrík, 2008; Jacobson & Gao, 2009). It has been found recently that adenosine plays an important role in anti-oxidative stress response in insects (Zemanová *et al.*, 2016), which is well known also for AKH (for review see Kodrík *et al.*, 2015)

2. AIMS

The aim of the bachelor thesis was to investigate if adipokinetic hormone (Drome-AKH) and adenosine control the activity of protease, amylase, and lipase in the fruit fly, *D. melanogaster.* To do this the Drosophila mutants with altered AKH production, AKH receptor site and adenosine receptor site were used. The effect of external application of Drome-AKH was studied as well.

3. MATERIALS AND METHODS

3.1 Chemicals used

3.1.1 Determination of amylases

1. DNS (3,5-dinitrosalicylic acid)
   - 0.0125 g DNS
   - 7.5 g K-Na tartare x 4 H_2O
   - 0.4 g NaOH

   Dissolved in 10ml of distilled water and filled up to 25 ml

2. 2% starch solution

3. Phosphate buffer (pH 5.7) + 20mM NaCl
   - 93.5 ml of KH_2PO_4 (2.722 g/100 ml)
   - 6.5 ml of Na_2HPO_4 (0.716 g/100 ml)

   To 100 ml of prepared mixture solution, 2.05 ml of 1 M NaCl (0.584 g/10 ml) were added

3.1.2 Determination of proteases

1. Substrate solution – 0.4% resorufin-casein in water

2. 0.2 M tris (pH 7.8)

3. 0.02 M $CaCl_2$

4. Blocking solution – 5% TCA (trichloroacetic acid)

3.1.3 Determination of lipases

1. 0.2 M tris (pH 7.8)

2. 50 mM 4-methylumbelliferyl butyrate (4MU-butyrate)

   - 0.0123 g of the substrate is dissolved in 1 ml dimethylsulfoxid (DMSO). 40µl aliquots of the solution are kept in dark eppendorf tubes at 20°C.

3.2 Experimental insect

Drosophila melanogaster

The experimental insect was kept in glass vials during the whole life cycle. Glass vials contained standard diet (corn meal/yeast/sucrose/agar) and were stored in thermostat at temperature 25°C under photoperiodic regime (12 hours light, 12 hours dark). The life cycle of Drosophila takes approximately 10 – 14 days. Five days old larvae (third instar larvae) and 5 days old adult flies were subjected to hormonal treatment and further analysis, without consideration of the gender.

Six different mutant lines were used for the experiments – White, AKH-deficient (AKH-def), AKHR-deficient (AKHR-def), AdoR-deficient (AdoR-def), AKH-rescue and Double mutant:

1. **White control (Bloomington #1118)** - served as control for other lines
2. **AKH-def** – mutant with production deficiency or more precisely with production of dysfunctional AKH. A mutation occurs in the gene, which regulates the synthesis of AKH, on chromosome III. The germline of this mutant was prepared by aimed mutation of the gene by TALEN (Transcription Activator-Like Effector Nuclease) method. The resulting genotype is: w; +; AKH-def / w; +; AKH-def. (Sajwan *et al.*, 2015)
3. **AKHR-def** – mutants missing genomic DNA sequences 2L 6711184 to 6716139, causing lack of the complete AKHR coding region. They were generated by a conventional P element–mobilization scheme The resulting genotype is: y* float w*; AKHR[1] / CyO float (Grönke *et al.*, 2007).
4. **AdoR-def** – mutant with dysfunctional adenosine receptor. A fly has a mutation in the gene for adenosine receptor on chromosome III. The resulting genotype is: w; +; AdoR-def / w; +; AdoR-def. Flies were prepared by homologous recombination (Doleželová *et al.*, 2007)
5. **AKH-rescue** – originally AKH-def mutant, in which the production of AKH was recovered. The mutant flies ectopically express AKH. The resulting genotype is: w; UAS – AKH^{+} = w^{+}; AKH-def / w; act – G4- w^{+}; AKH-def. (Sajwan *et al.*, 2015)
6. **Double mutant** – mutant with deficiency of production of AKH combined with dysfunctional receptor for adenosine. The double mutant was prepared by crossing of

homozygous strains of mutants in the adenosine receptor and adipokinetic hormone and making a recombinant (Zemanová *et al.*, 2016).

3.3 Hormonal treatment

D. melanogaster AKH (Drome-AKH; pGlu-Leu-Thr-Phe-Ser-Pro-Asp-Trp-NH_2) was applied to experimental flies and larvae. For the application, flies were immobilized by coldness and shortly immersed in hormone solved in 20% methanol in Ringer saline (final concentration 3 pmol/µl). The controls were immersed in the solvent only. After immersion, flies were left to dry on a paper attached to cooling device and subsequently transferred to glass vials, where they were left for 2 hours. Next, they were killed by freezing and left in the freezer before further use.

3.4 Determination of enzyme activity

The activities of amylases and lipases were determined in the whole body of adult fly and larvae (third instar). Protease activity was determined in the whole larval bodies (third instar) only.

The activity of amylase was assessed according to Bernfeld (1955) as modified by Kodrík *et al.* (2012). Protease activity was determined according to Roche and lipase activity according to Roberts (1985) as modified by Kodrík e*t al.* (2012).

3.4.1 Amylase activity

3.4.1.1 Principle of the method

Starch molecules contain α-1,4 glycosidic bonds, which can be cleaved hydrolytically in reaction catalysed by amylases. Hydrolysis of starch results in the formation of maltose (reducing sugar), and other products. The concentration of maltose is measured by its ability to reduce 3,5-dinitrosalicylic acid (DNS) into 3-amino-5-nitrosalicylic acid (ANS), which strongly absorbs light at 540 nm.

3.4.1.2 Sample preparation

For the preparation of one sample, 4 adult flies or larvae were homogenized (ULTRASONIC HOMOGENIZER 4710 series) in 50µl of 100 mM phosphate buffer. The homogenate was centrifuged and 25µl aliquot (= 2 flies/larvae equiv.) was mixed with an equal volume of 2% soluble starch made of the same buffer with 40 mM NaCl. The reaction mixture was

incubated at 30°C for 1 hour while shaken, followed by addition of 200µl of DNS reagent. The reaction was terminated by heating at 100°C for 5 minutes. Next, the solution was centrifuged for 10 minutes at 10000 rpm and the absorbance was measured in 200µl of the supernatant at 550nm. Controls without sample were measured simultaneously. Enzyme activity was calculated in µmol of maltose per whole body.

3.4.2 Protease activity

3.4.2.1 Principle of the method

Phosphoprotein casein contains peptides, which can be labelled by resorufin. When such labelled peptides get in contact with proteases, they are released from casein and cannot be precipitated when subsequently treated with trichloroacetic acid. The concentration of these non-precipitated peptides in the supernatant corresponds to the activity of proteases.

3.4.2.2 Sample preparation

For determination of protease activity in the whole larval body, one sample was prepared from 5 larvae in 50µl of 0.2 M tris buffer (pH 7.8) - to obtain final concentration 2 equiv./20µl. The sample was homogenized by sonicator and then centrifuged. Next, reaction mixture was prepared by mixing 20µl of resorufin-casein in water, 40µl of tris buffer, 20µl of $CaCl_2$ solution and 20µl of supernatant of the sample extract. The reaction mixture was incubated for 1 hour at 37°C while gentle shaking. The reaction was terminated by addition of 240µl TCA and incubation at 37°C for another 10 minutes. The solution was subsequently centrifuged for 3 minutes at 10000 rpm. The absorbance was measured in 300µl of the supernatant at 490nm. At the same time, the controls were assayed. Protease activity was determined as a relative absorbance per whole larval body.

3.4.3 Lipase activity

3.4.3.1 Principle of the method

4-methylumbelliferyl butyrate is non-fluorescent compound which contains one fatty acid ester bond. Hydrolysis of this bond yields one molecule of highly fluorescent compound 4-methylumbellifrone (4-MU) and one molecule of butyric acid. The reaction is catalysed by digestive lipases, therefore changes in fluorescence correspond to lipase concentration and activity. (Roberts, 1985)

3.4.3.2 Sample preparation

For the lipase activity determination the working concentration of the substrate (2mM 4MU-butyrate) was prepared by 25-fold dilution of the stock solution (50mM) by DMSO.

The lipase extract was prepared from one whole body (adult fly or larva) homogenized in 40µl of 0.2 M tris buffer (pH 7.8). Five µl of this extract was added to next 195µl of the tris buffer in the microplate wells. Then, 5 µl of the substrate (2mM 4MU-butyrate) was added, and samples were incubated at 30°C directly in the measuring device (Synergy 4 multi-mode microplate reader /BioTek Instr., Winooski, Vermont). The release of 4MU was monitored at 5 min intervals utilizing excitation wavelength 327 nm and emission wavelength 449 nm. Corresponding blanks were measured simultaneously with the sample. Lipase activity was expressed in pmol of 4-MU/min/1 whole body.

3.5 Statistics

Results were processed by Microsoft Excel 2010 software and graphs were plotted using the graphic software Prism (Graph Pad Software, version 6.0, San Diego, CA, USA). The bar graphs represent mean ± standard deviation. Statistically significant differences (5% significance level) were evaluated by one-way ANOVA with Dunnett's post test using the Prism software.

For the experiments two groups of flies were used:

1. **Control group** - group of subjects with no AKH treatment
2. **Hormone group** – group of subjects treated with AKH solved in 20% methanol in Ringer saline.

For each group, the number of replicates n=8.

4. RESULTS

4.1 Activity of enzymes in larvae

In the first series of measurements, activities of enzymes were determined in larvae. The results showed that protease activity in AKH-deficient larvae was significantly lower (2.1 times) and in AKH-rescue larvae significantly higher (1.6 times) than in the White control group. Protease activity in AdoR-deficient line was 1.4 times lower than the activity in White control group, however, the difference was not significant (Figure 2).

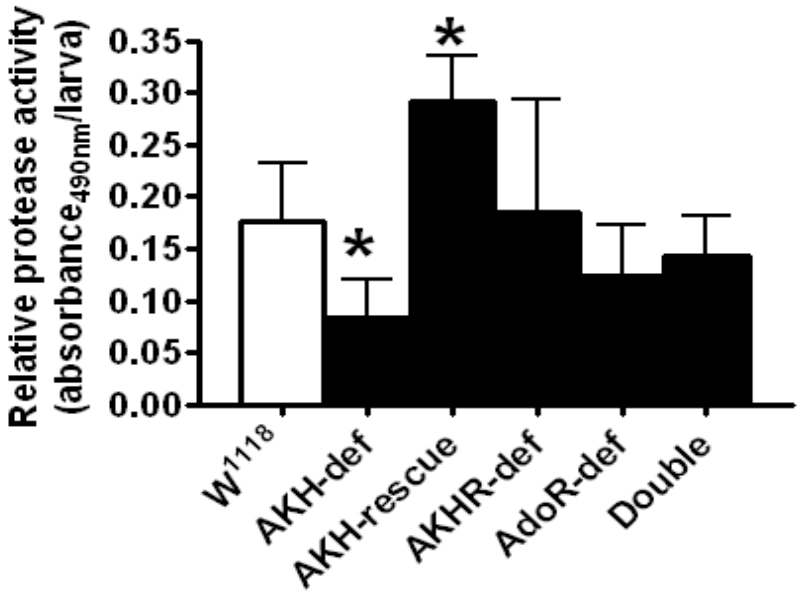

Figure 2: Relative protease activity (absorbance$_{490nm}$/larva) in different mutant lines (average ± SD, n=8). Statistically significant differences between line White and other lines were evaluated by one-way ANOVA with Dunnett's post test (5% significance level) and are marked with *.

Amylase activity did not differ significantly among the lines. The AKH-deficient line and the Double line showed lower activity (1.4 times lower for both lines) than the White control line, but the difference was not significant. Values of the AKHR-deficient line were excluded from the Figure 3 due to the inconsistency of the results.

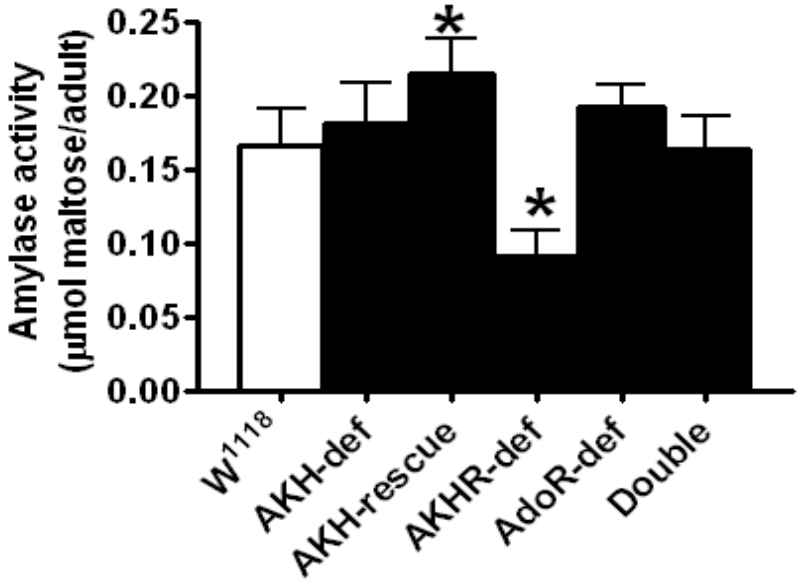

Figure 3: Amylase activity (µmol maltose/larva) in different mutant lines (average ± SD, n=8). Statistically significant differences between line White and other lines were evaluated by one-way ANOVA with Dunnett's post test (5% significance level).

Significant differences of lipase activity were observed in the AKH-rescue and Double lines when compared with the White line (Figure 4). In both cases the activity was higher, in AKH-rescue 1.6 times and in Double 1.7 times. Other lines showed no significant difference in comparison with the White line.

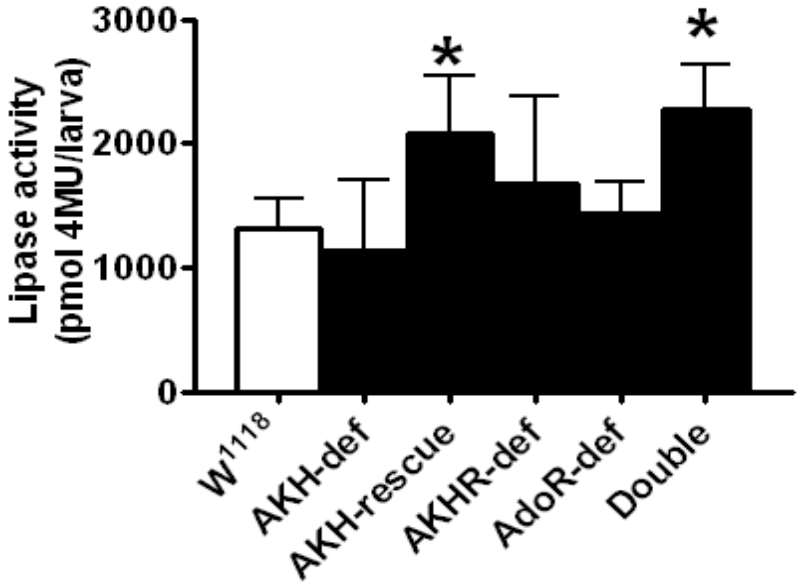

Figure 4: Lipase activity (pmol 4MU/larva) in different mutant lines (average ± SD, n=8). Statistically significant differences between line White and other lines were evaluated by one-way ANOVA with Dunnett's post test (5% significance level) and are marked with *.

4.2 Activity of enzymes in adult flies

In the second series of measurements enzymatic activities were determined in adult flies. Amylase activity was significantly higher in AKH-rescue flies (1.3 times) and significantly lower in AKHR-deficient flies (1.5 times) when compared with White flies (Figure 5).

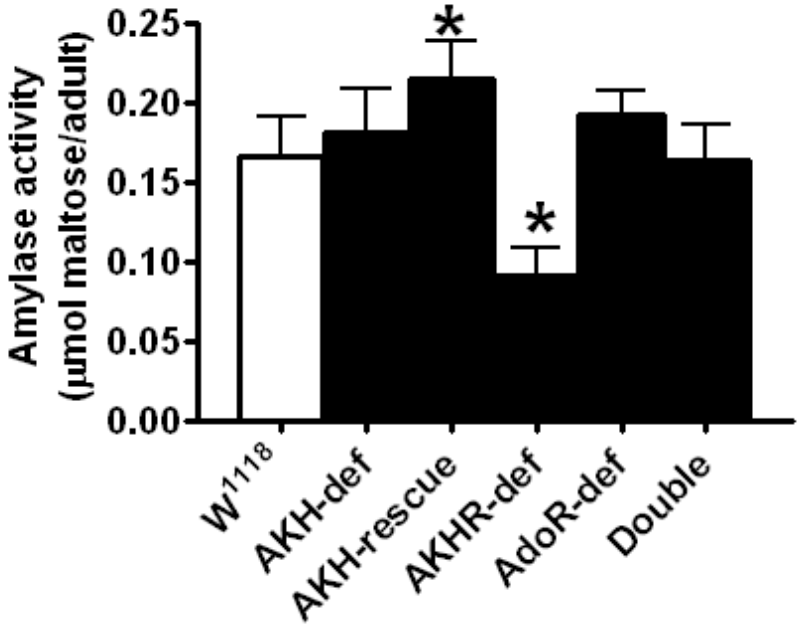

Figure 5: Amylase activity (µmol maltose/adult) in different mutant lines (average ± SD, n=8). Statistically significant differences between line White and other lines were evaluated by one-way ANOVA with Dunnett's post test (5% significance level) and are marked with *.

Measurements of lipase activity revealed significantly higher values in AKHR-def (1.7 times), AdoR-deficient (2.3 times) and Double (1.6 times)adult flies than in White flies (Figure 6).

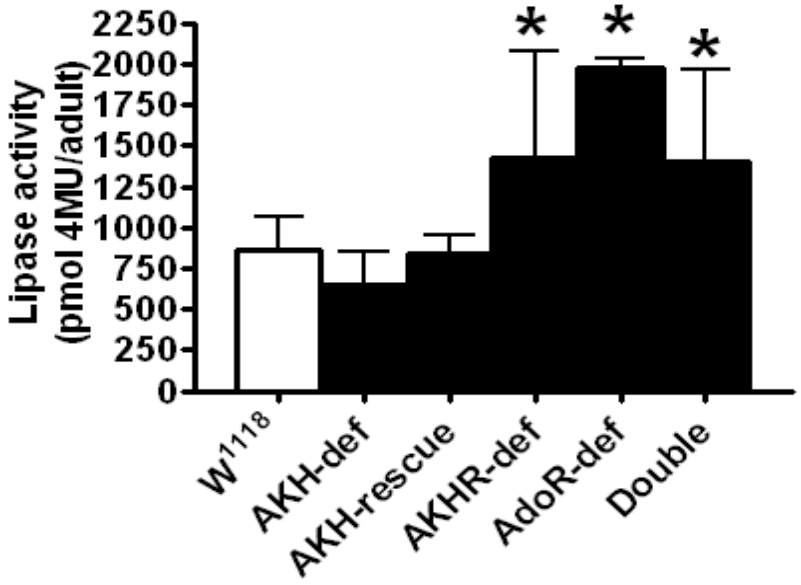

Figure 6: Lipase activity (pmol 4MU/adult) in different mutant lines (average ± SD, n=8). Statistically significant differences between line White and other lines were evaluated by one-way ANOVA with Dunnett's post test (5% significance level) and are marked with *.

4.3 Activity of enzymes in larvae treated with hormone (Drome-AKH)

In the following sets of measurements, the effect of applied Drome-AKH was studied in larvae. In most lines, the application of Drome-AKH did not affect the protease activity significantly (Figure 7). However, significant increase was observed in the AKH-deficient line (4.9 times) and significant decrease of protease activity was observed in the AKH-rescue line (1.3 times).

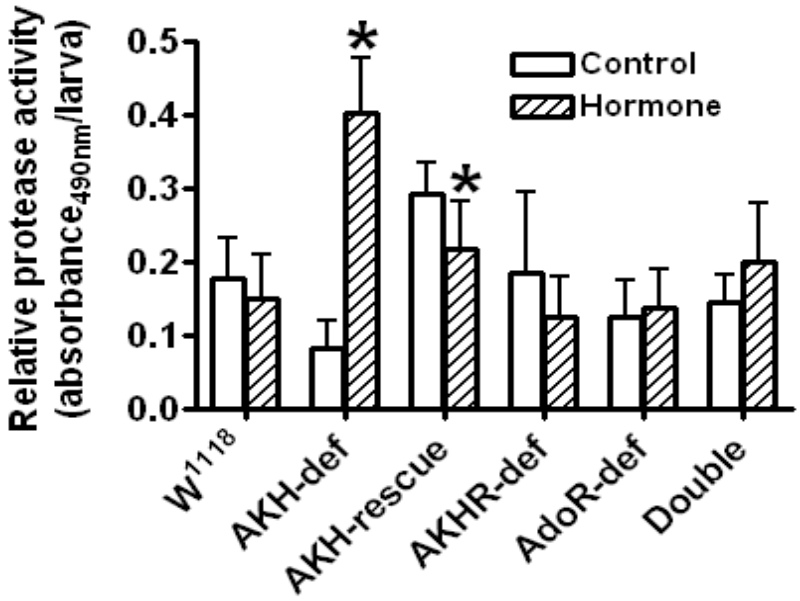

Figure 7: Relative protease activity (absorbance$_{490nm}$/larva) in different mutant lines treated with hormone (Drome-AKH) together with control groups without treatment (average ± SD, n=8). Statistically significant differences between hormonally treated groups and relevant control groups were evaluated by unpaired Student's t-test (5% significance level) and are marked with *.

Amylase activity was increased in most lines after the application of Drome-AKH (Figure 8). Significant increase was observed in the White group (1.8 times), the AKH-deficient group (2.7 times), the AdoR-deficient group (2.2 times) and in the Double group (1.3 times).

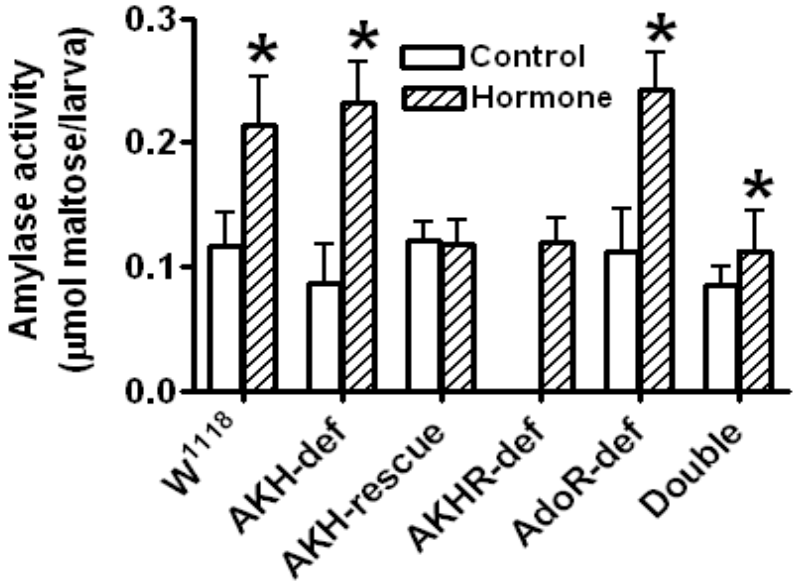

Figure 8: Amylase activity (µmol maltose/larva) in different mutant lines treated with hormone (Drome-AKH) together with control groups without treatment (average ± SD, n=8). Statistically significant differences between hormonally treated groups and relevant control groups were evaluated by unpaired Student's t-test (5% significance level) and are marked with *.

Effect of Drome-AKH on lipase activity was recorded to be significant only in two lines – the AKHR-deficient and the Double line (Figure 9). In both cases Drome-AKH significantly inhibited the activity of lipase: 1.8 times in the AKHR-deficient and 1.4 times in the Double line.

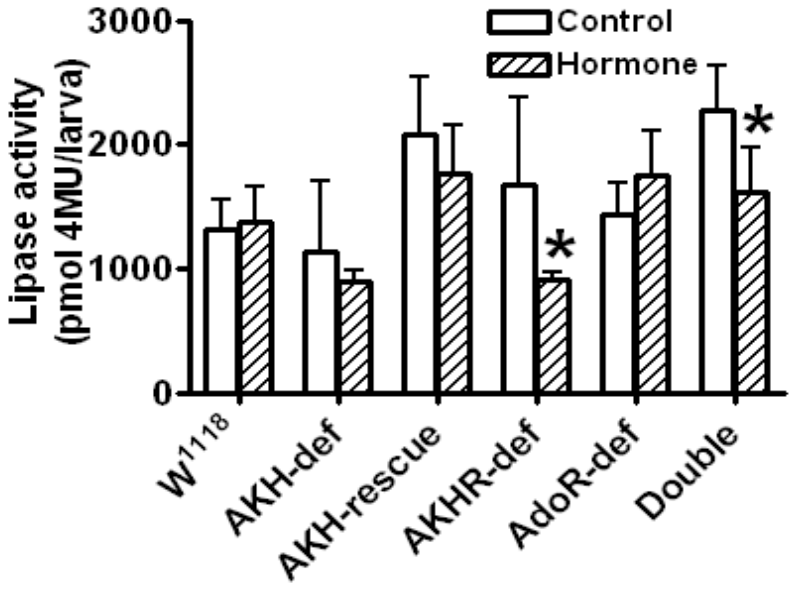

Figure 9: Lipase activity (pmol 4MU/larva) in different mutant lines treated with hormone (Drome-AKH) together with control groups without treatment (average ± SD, n=8). Statistically significant differences between hormonally treated groups and relevant control groups were evaluated by unpaired Student's t-test (5% significance level) and are marked with *.

4.4 Activity of enzymes in adult flies treated with hormone (Drome-AKH)

In the last series of measurements, the effect of applied Drome-AKH hormone in adult flies was studied. The results revealed that amylase activity in the AKH-rescue group, the AdoR-deficient group and in the Double group after the treatment increased slightly, but significantly 1.2 times, 1.2 times and 1.3 times respectively (Figure 10).

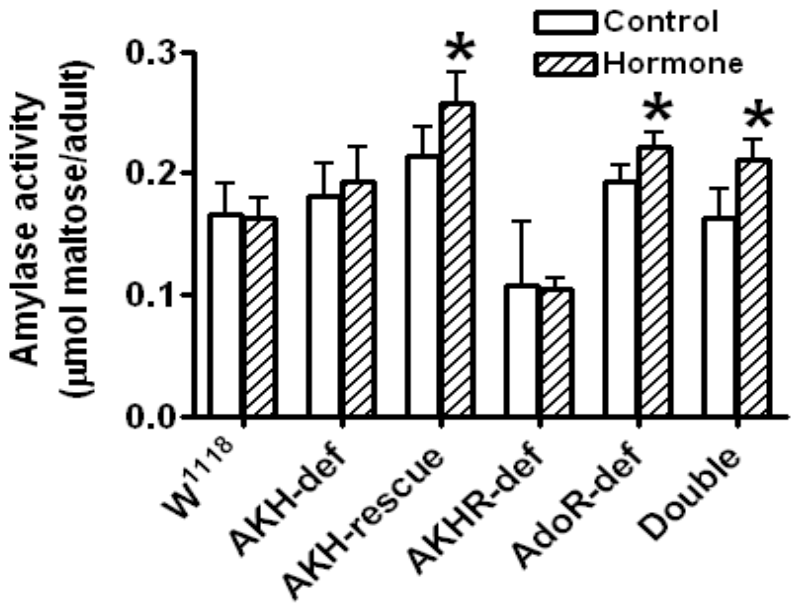

Figure 10: Amylase activity (µmol maltose/adult) in different mutant lines treated with hormone (Drome-AKH) together with control groups without treatment (average ± SD, n=8). Statistically significant differences between hormonally treated groups and relevant control groups were evaluated by unpaired Student's t-test (5% significance level) and are marked with *.

Treatment with Drome-AKH significantly affected lipase activity in three groups (Figure 11). Decrease of activity was observed in the AKHR-deficient line (2.7 times) and in the AdoR-deficient line (6.4 times). On the other hand, in the AKH-deficient line a significant increase (1.4 times) was observed.

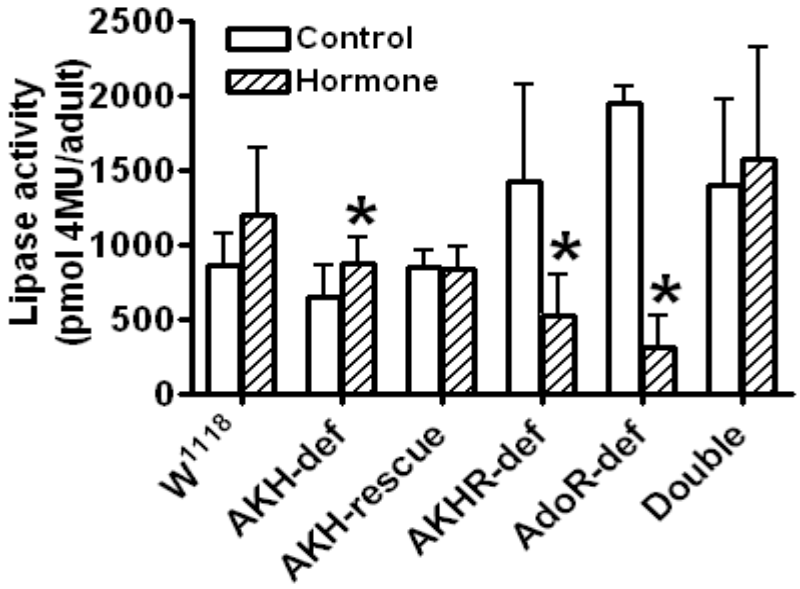

Figure 11: Lipase activity (pmol 4MU/adult) in different mutant lines treated with hormone (Drome-AKH) together with control groups without treatment (average ± SD, n=8). Statistically significant differences between hormonally treated groups and relevant control groups were evaluated by unpaired Student's t-test (5% significance level) and are marked with *.

5. DISCUSSION

The main goal of this thesis was to do a basic screening of the activity of Drosophila elementary digestive enzymes (proteases, amylases, lipases) in the lines with dysfunctional production of AKH, and dysfunctional AKH and adenosine receptors to judge the possible effect of those two agents - AKH and adenosine - on digestive processes. As expected the results were variable showing that this phenomenon is a complicated matter; and further, it is clearly evident that many experiments must still be done for better understanding of the topic. Anyway, the results revealed no uniform trend, especially if both, adult flies and larvae were taken into account simultaneously. However, as expected, in some genetically manipulated lines AKH showed a significant effect on the stimulation of digestive enzymes activity. The activity of all studied enzymes was lower in larvae producing non-functional Drome-AKH (AKH-def) than in the control (White). Specifically, the protease activity decreased significantly and the amylase activity also decreased evidently (just slightly below the significance level) in this line, however, the effect seemed to be the least pronounced in the lipase activity. A lower effect of AKH on lipase activity might be explained by a lower level of lipids in the food and thus lower importance of lipases in Drosophila digestion; whether there are some other metabolic reasons remains to be speculation. The mentioned effect of AKH on amylase activity is in accordance with the findings of Sajwan *et al.* (2015), who recorded lowered levels of total carbohydrates and trehalose in larval AKH-def line. In larvae with ectopically expressed AKH (AKH-rescue), the activities of lipase and protease were significantly increased, whereas the activity of amylase was not changed in comparison to the control.

Regarding the effect of adenosine, the activity of lipase and amylase in larval gut did not change and protease activity decreased minimally as observed in the larvae with a mutation in the adenosine receptor gene (AdoR-def). It seems that adenosine plays no role in modulation of at least larval digestion.

The amylase and lipase activity did not change significantly in Drosophila adults of AKH-def line and concerning AKH-rescue line, a significant increase was observed only in the amylase activity. It is interesting that the activity of both enzymes increased in AdoR-def flies, however, only lipase activity increased significantly. That suggests inhibiting role of adenosine. Uniform trend was found in mutants with deficiency of production of AKH combined with dysfunctional receptor for adenosine (Double), and in mutants with

dysfunctional AKH receptor (AKHR-def) while studying the lipase activity. In the former, for both, larvae and adult flies, the activity increased significantly. That possibly proves the inhibiting effect of adenosine on the lipase activity, since neither larvae, nor adults only with deficiency of production of AKH showed increase in the activity before. The latter, AKHR line, showed significant increase only in the adults, however the activity increased minimally in larvae as well. To sum up those rather heterogeneous set of results of enzymatic activities in adult gut, one can conclude that the role of AKH and adenosine in control of the Drosophila adult digestion is somewhat unclear. Some results suggest a modulatory effect of both, or even their mutual cooperation, however, other ones do not support this suggestion. Nevertheless, without additional experiments a definite conclusion cannot be done.

Nor did experiments with externally applied Drome-AKH solve the complicated situation, however, they confirmed the literature data that AKH has more or less evident effect on stimulation of the digestive enzymes. As expected, the protease and amylase activity in the AKH-def larvae increased significantly. Since *D. melanogaster* prefers carbohydrate metabolism, it is not surprising that the activity of amylase increased significantly after the application of Drome-AKH in all lines in larvae except the AKH-rescue line. This line showed no change in the activity of all studied enzymes after the application, with the exception of the amylase activity in the adult flies, which increased significantly. The reason behind such ambiguous behaviour can be either too high level of natural Drome-AKH (in fact this line is not only AKH 'rescue', but even AKH 'overexpressed' with higher AKH level than in control White mutant – see Zemanová *et al.,* (2016) or the complicated genetic background of this mutant – it was obtained by crossing of AKH-def mutant and furthermore ectopical overexpression of AKH was induced (for details see Sajwan *et al.*, 2015). Treatment of adult flies with Drome-AKH also increased the amylase activity in most lines, except for White and AKHR-def line.

Despite certain ambiguities mentioned above, the obtained results fit into the generally accepted stimulatory effect of AKH on insect digestion. The first indications that AKH might affect gut functions resulted from studies demonstrating the expression of AKH receptor in the insect gut (*Periplaneta americana*, Wicher *et al.,* 2006; *Manduca sexta,* Ziegler *et al.,* 2011; *Blatella germanica*, Huang *et al.,* 2012). However, the first evidence describing AKH's role in digestion was obtained from the firebug *Pyrrhocoris apterus*, where the injection of Pyrap-AKH significantly stimulated peptidase and alpha-glucosidase activities in its midgut

(Kodrík *et al.,* 2012). The stimulatory effect of AKH on the gut digestive enzymes was also recorded by Bil *et al.* (2014) in *Sarcophaga crassipalpis*. These authors found that injection of AKH into decapitated flies or sugar-fed intact flies enhanced midgut proteolytic activity up to the level found in intact flies. In recent studies (Bodláková *et al.,* 2017; Bodláková *et al*., submitted), it has been found that injection of AKH into the cockroach *P. americana* body significantly stimulated the main gut digestive enzymes, and in addition that *in vitro* experiments, where application of AKHs to the medium had the same effect. This suggested direct hormonal stimulation of enzyme activities not mediated by other organs or inter-organ cascades. The interesting role of AKH in Drosophila digestion has been recently described by Gáliková *et al.* (2017), who reported Drome-AKH increased food intake in these flies, despite its anti-obesity function. Both effects are likely to be controlled by AKH independently, but the mechanism underlying this control might be complicated because Drome-AKH also regulates the expression of orexigenic (= appetite stimulating) factors and metabolic hormones, including corazonin and insulin-like peptides (Gáliková *et. al.,* 2017).

As far as I know, no literature data is available about the role of adenosine in the activity of digestive enzymes in insects. Also, the data of this thesis suggests that adenosine involvement is probably not important, nevertheless, some minor role cannot be completely excluded. However, without additional data it remains to be speculation.

6. CONCLUSION

The experiments confirmed that adipokinetic hormone plays a role in the control of the activity of digestive enzymes. Although no uniform trend in the stimulation was observed, in some mutant lines the gene manipulations showed AKH effect on the activity of proteases and amylases. Adenosine was observed to have no or just minor, probably inhibitory effect on enzymatic activities. Externally applied Drome-AKH revealed stimulating effect on the amylase and partially protease activities.

7. REFERENCES

Arrese, E. L., Canavoso, L. E., Jouni, Z. E., Pennington, J. E., Tsuchida, K., & Wells, M. A. (2001). Lipid storage and mobilization in insects: current status and future directions. *Insect Biochemistry and Molecular Biology*, *31*(1), 7-17.

Baldwin, S. A., Beal, P. R., Yao, S. Y., King, A. E., Cass, C. E., & Young, J. D. (2004). The equilibrative nucleoside transporter family, SLC29. *Pflügers Archiv: European Journal of Physiology*,*447*(5), 735-743.

Bernfeld, P. (1955) Amylases, α and β. *Methods in Enzymology*, (ed. by Colowick, S.P. and Kaplan, N.O.), Vol. 1, pp. 149-158, Academic Press, New York.

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. *General and Comparative Endocrinology*, *208*, 49-56.

Bodláková, K., Beňová, M., & Kodrík, D. (2017). The effect of adipokinetic hormones on the activity of digestive enzymes in insects. *Manuscript submitted for publication*

Bodláková, K., Jedlička, P., & Kodrík, D. (2017). Adipokinetic hormones control amylase activity in the cockroach (Periplaneta americana) gut. *Insect science*, *24*(2), 259-269.

Carlisle, J. A., & Loughton, B. G. (1979). Adipokinetic hormone inhibits protein synthesis in Locusta. *Nature*, *282*(5737), 420-421.

Chapman, R. F. (1998). *The Insects: Structure and Function*, 4th ed., Cambridge university press, Cambridge

Doleželová, E., Nothacker, H. P., Civelli, O., Bryant, P. J., & Žurovec, M. (2007). A Drosophila adenosine receptor activates cAMP and calcium signaling. *Insect Biochemistry and Molecular Biology*, *37*(4), 318-329.

Fredholm, B. B. (2010). Adenosine receptors as drug targets. *Experimental Cell Research*, *316*(8), 1284-1288.

Gáliková, M., Klepsatel, P., Xu, Y., & Kühnlein, R. P. (2017). The obesity-related Adipokinetic hormone controls feeding and expression of neuropeptide regulators of Drosophila metabolism. *European Journal of Lipid Science and Technology*, *119*(3).

Gäde, G. (2009). Peptides of the Adipokinetic Hormone/Red Pigment-Concentrating Hormone Family. *Annals of the New York Academy of Sciences*,*1163*(1), 125-136.

Gäde, G., & Auerswald, L. (2002). Beetles' choice—proline for energy output: control by AKHs. *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology*, *132*(1), 117-129.

Gäde, G., & Auerswald, L. (2003). Mode of action of neuropeptides from the adipokinetic hormone family. *General and Comparative Endocrinology*, *132*(1), 10-20.

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*(10), 1063-1075.

Gäde, G., Hoffmann, K. H., & Spring, J. H. (1997). Hormonal regulation in insects: facts, gaps, and future directions. *Physiological Reviews*, *77*(4), 963-1032.

Gokuldas, M., Hunt, P. A., & Candy, D. J. (1988). The inhibition of lipid synthesis in vitro in the locust, Schistocerca gregaria, by factors from the corpora cardiaca. *Physiological Entomology*, *13*(1), 43-48.

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

Goldsworthy, G., Opoku-Ware, K., & Mullen, L. (2002). Adipokinetic hormone enhances laminarin and bacterial lipopolysaccharide-induced activation of the prophenoloxidase cascade in the African migratory locust, Locusta migratoria. *Journal of Insect Physiology*, *48*(6), 601-608.

Gray, J. H., Owen, R. P., & Giacomini, K. M. (2004). The concentrative nucleoside transporter family, SLC28. *Pflügers Archiv: European Journal of Physiology*, *447*(5), 728-734.

Grönke, S., Müller, G., Hirsch, J., Fellert, S., Andreou, A., Haase, T., ... & Kühnlein, R. P. (2007). Dual lipolytic control of body fat storage and mobilization in Drosophila. *PLoS biology*, *5*(6), e137.

Gullan, P. J., & Cranston, P. S. (2005). *The Insects: An Outline of Entomology*, 3rd ed. John Wiley & Sons.

Haskó, G., Deitch, E. A., Szabó, C., Németh, Z. H., & Vizi, E. S. (2002). Adenosine: a potential mediator of immunosuppression in multiple organ failure. *Current Opinion in Pharmacology*, *2*(4), 440-444.

Huang, J. H., Bellés, X., & Lee, H. J. (2012). Functional characterization of hypertrehalosemic hormone receptor in relation to hemolymph trehalose and to oxidative stress in the cockroach Blattella germanica. *Frontiers in endocrinology*, *2*.

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

Kanost, M. R., Kawooya, J. K., Law, J. H., Ryan, R. O., Van Heusden, M. C., & Ziegler, R. (1990). Insect haemolymph proteins. *Advances in Insect Physiology*, *22*, 299-396.

Keeley, L. L., Hayes, T. K., Bradfield, J. Y., & Sowa, S. M. (1991). Physiological actions by hypertrehalosemic hormone and adipokinetic peptides in adult Blaberus discoidalis cockroaches. *Insect Biochemistry*, *21*(2), 121-129.

Keeley, L. L., Park, J. H., Lu, K. H., & Bradfield, J. Y. (1996). Neurohormone signal transduction for dual regulation of metabolism and gene expression in insects: Hypertrehalosemic hormone as a model. *Archives of Insect Biochemistry and Physiology*, *33*(3-4), 283-301.

Kodrík, D. (2008). Adipokinetic hormone functions that are not associated with insect flight. *Physiological Entomology*, *33*(3), 171-180.

Kodrík, D., & Goldsworthy, G. J. (1995). Inhibition of RNA synthesis by adipokinetic hormones and brain factor (s) in adult fat body of Locusta migratoria. *Journal of Insect Physiology*, *41*(2), 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*(5), 974-980.

Kodrík, D., Bednářová, A., Zemanová, M., & Krishnan, N. (2015). Hormonal regulation of response to oxidative stress in insects—an update. *International Journal of Molecular Sciences*, *16*(10), 25788-25816.

Kodrík, D., Vinokurov, K., Tomčala, A., & Socha, R. (2012). The effect of adipokinetic hormone on midgut characteristics in Pyrrhocoris apterus L. (Heteroptera). *Journal of Insect Physiology*, *58*(1), 194-204.

Kodrik, D., Marco, H. G., Šimek, P., Socha, R., Štys, P., & Gaede, G. (2010). The adipokinetic hormones of Heteroptera: a comparative study. *Physiological Entomology*, *35*(2), 117-127.

Köllisch, G. V., Lorenz, M. W., Kellner, R., Verhaert, P. D., & Hoffmann, K. H. (2000). Structure elucidation and biological activity of an unusual adipokinetic hormone from corpora cardiaca of the butterfly, Vanessa cardui. *The FEBS Journal*, *267*(17), 5502-5508.

Kučerová, L., Broz, V., Fleischmannová, J., Santrucková, E., Sidorov, R., Doležal, V., & Žurovec, M. (2012). Characterization of the Drosophila adenosine receptor: the effect of adenosine analogs on cAMP signaling in Drosophila cells and their utility for in vivo experiments. *Journal of Neurochemistry*, *121*(3), 383-395.

Lee, G., & Park, J. H. (2004). Hemolymph sugar homeostasis and starvation-induced hyperactivity affected by genetic manipulations of the adipokinetic hormone-encoding gene in Drosophila melanogaster. *Genetics*, *167*(1), 311-323.

Lemaitre, B., & Miguel-Aliaga, I. (2013). The digestive tract of Drosophila melanogaster. *Annual Review of Genetics*, *47*, 377-404.

Lorenz, M. W. (2003). Adipokinetic hormone inhibits the formation of energy stores and egg production in the cricket Gryllus bimaculatus. *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology*,*136*(2), 197-206.

Lloyd, H. G. E., & Fredholm, B. B. (1995). Involvement of adenosine deaminase and adenosine kinase in regulating extracellular adenosine concentration in rat hippocampal slices. *Neurochemistry International*, *26*(4), 387-395.

Molina-Arcas, M., Casado, F. J., & Pastor-Anglada, M. (2009). Nucleoside transporter proteins. *Current Vascular Pharmacology*, *7*(4), 426-434.

Noyes, B. E., Katz, F. N., & Schaffer, M. H. (1995). Identification and expression of the Drosophila adipokinetic hormone gene. *Molecular and Cellular Endocrinology*, *109*(2), 133-141.

O'Shea, M., Witten, J., & Schaffer, M. (1984). Isolation and characterization of two myoactive neuropeptides: further evidence of an invertebrate peptide family. *Journal of Neuroscience*, *4*(2), 521-529.

Park, Y., Kim, Y. J., & Adams, M. E. (2002). Identification of G protein-coupled receptors for Drosophila PRXamide peptides, CCAP, corazonin, and AKH supports a theory of ligand-receptor coevolution. *Proceedings of the National Academy of Sciences*, *99*(17), 11423-11428.

Roberts, I. M. (1985). Hydrolysis of 4-methylumbelliferyl butyrate: a convenient and sensitive fluorescent assay for lipase activity. *Lipids*, *20*(4), 243-247.

Rose, J. B., & Coe, I. R. (2008). Physiology of nucleoside transporters: back to the future.... *Physiology*, *23*(1), 41-48.

Sajwan, S., Sidorov, R., Stašková, T., Žaloudíková, A., Takasu, Y., Kodrík, D., & Zurovec, M. (2015). Targeted mutagenesis and functional analysis of adipokinetic hormone-encoding gene in Drosophila. *Insect Biochemistry and Molecular Biology*, *61*, 79-86.

Scarborough, R. M., Jamieson, G. C., Kalish, F., Kramer, S. J., McEnroe, G. A., Miller, C. A., & Schooley, D. A. (1984). Isolation and primary structure of two peptides with cardioacceleratory and hyperglycemic activity from the corpora cardiaca of Periplaneta americana. *Proceedings of the National Academy of Sciences*, *81*(17), 5575-5579.

Schaffer, M. H., Noyes, B. E., Slaughter, C. A., Thorne, G. C., & Gaskell, S. J. (1990). The fruitfly Drosophila melanogaster contains a novel charged adipokinetic-hormone-family peptide. *Biochemical Journal*, *269*(2), 315-320.

Socha, R., Kodrík, D., & Zemek, R. (1999). Adipokinetic hormone stimulates insect locomotor activity. *Naturwissenschaften*, *86*(2), 85-86.

Spencer, I. M., & Candy, D. J. (1976). Hormonal control of diacyl glycerol mobilization from fat body of the desert locust, Schistocerca gregaria. *Insect Biochemistry*, *6*(3), 289-296.

Spit, J., Badisco, L., Verlinden, H., Van Wielendaele, P., Zels, S., Dillen, S., & Vanden Broeck, J. (2012). Peptidergic control of food intake and digestion in insects 1 1 This review

is part of a virtual symposium on recent advances in understanding a variety of complex regulatory processes in insect physiology and endocrinology, including development, metabolism, cold hardiness, food intake and digestion, and diuresis, through the use of omics technologies in the postgenomic era. *Canadian Journal of Zoology*, *90*(4), 489-506.

Stone, J. V., Mordue, W., Batley, K. E., & Morris, H. R. (1976). Structure of locust adipokinetic hormone, a neurohormone that regulates lipid utilisation during flight. *Nature*, *263*(5574), 207-211.

Van Der Horst, D. J., Van Marrewijk, W. J. A., Vullings, H. G., & Diederen, J. H. (1999). Metabolic neurohormones: release, signal transduction and physiological responses of adipokinetic hormones in insects. *European Journal of Entomology*, *96*, 299-308.

Van Marrewijk, W. J. A., Van den Broek, A. T. M., & Beenakkers, A. T. (1980). Regulation of glycogenolysis in the locust fat body during flight. *Insect Biochemistry*, *10*(6), 675-679.

Vroemen, S. F., Van Marrewijk, W. J., De Meijer, J., Van den Broek, A. T. M., & Van der Horst, D. J. (1997). Differential induction of inositol phosphate metabolism by three adipokinetic hormones. *Molecular and cellular endocrinology*, *130*(1), 131-139.

Wicher, D., Söhler, S., Gundel, M., Heinemann, S. H., Wollweber, L., Stengl, M., & Derst, C. (2006). Differential receptor activation by cockroach adipokinetic hormones produces differential effects on ion currents, neuronal activity, and locomotion. *Journal of Neurophysiology*, *95*(4), 2314-2325.

Zemanová, M., Stašková, T., & Kodrík, D. (2016). Role of adipokinetic hormone and adenosine in the anti-stress response in Drosophila melanogaster. *Journal of Insect Physiology*, *91*, 39-47.

Ziegler, R., Isoe, J., Moore, W., Riehle, M. A., & Wells, M. A. (2011). The putative AKH receptor of the tobacco hornworm, Manduca sexta, and its expression. *Journal of Insect Science*, *11*(1).

8. APPENDIX

Selected results of this thesis are part of the manuscript submitted to the *Physiological Entomology* journal:

The effect of adipokinetic hormones on the activity of digestive enzymes in insects

KAROLINA BODLÁKOVÁ[1], MICHAELA BEŇOVÁ[2] and DALIBOR KODRÍK[2]

[1]Gymnasium, Česká 64, 370 21 České Budějovice, Czech Republic

[2]Institute of Entomology, Biology Centre, CAS, and Faculty of Science, University of South Bohemia, Branišovská 31, 370 05 České Budějovice, Czech Republic

Correspondence: Dalibor Kodrík, Institute of Entomology, BC CAS, Branišovská 31, 370 05 České Budějovice, Czech Republic Tel.: +420 387 775 271; fax: +420 385 310 254; email: kodrik@entu.cas.cz

Short title: Effect of AKH on digestion

Abstract. The role of the adipokinetic hormone (AKH) in the control of protease, amylase, and lipase activities was examined using the cockroach *Periplaneta americana* and the fruit fly *Drosophila melanogaster* as model species. The effects of Peram-CAH-I and -II on the activity of cockroach digestive enzymes in gastric caeca and midgut itself were measured both *in vivo* and *in vitro*. The results proved activity of proteases, amylases and lipases in both portions of the gut, however, amylase activity was higher in the gastric caeca than in the midgut, lipase activity presented the opposite trend, and protease activity was similar in both organs. The applied hormones stimulated the activity of all digestive enzymes, but this stimulation was not uniform: AKHs affected enzymes selectively, and in some cases unequally, in the gastric caeca and midgut. Still, no substantial differences between Peram-CAH-I and -II stimulation were recorded. *In vitro* results demonstrated the direct AKH stimulation of digestive enzyme activity in insect gut. In agreement with the cockroach results, enzymatic activity in *D. melanogaster* larvae producing non-functional AKH was lower than in the larvae with ectopically expressed *Akh* gene, where enzyme activity reached or even exceeded that of controls. Overall, the results demonstrated the active role of AKHs in the stimulation of digestive enzymes activity.

Keywords. Adipokinetic hormone, AKH, amylase, digestion, *Drosophila Akh* mutant, enzyme, lipase, midgut, *Periplaneta* cockroach, protease

Introduction

The digestive system of insects provides food intake and digestion as well as the absorption of the resulting nutrients. It is known to be under hormonal control, and many neuropeptide candidates have been suggested to control insect feeding and gut functions through their various activities, although general insect gut endocrinology is far from being understood. Nevertheless, proctolin, allatotropin, and tachykinins are thought to stimulate the gut muscles' activity, whereas allatostatins, myosuppressins, and myoinhibitory peptides inhibit it (Audsley & Weaver, 2009). Other neuropeptides often have contradictory functions: sulfakinins, for example, stimulated digestive functions by increasing gut contractions in the cockroach *Leucophora maderae* (Nachman et al., 1986a,b) but reduced digestive enzyme secretion in the midgut and gastric caeca of the migratory locust *Locusta migratoria* (Zels et

al., 2015). Allatostatins are known to stimulate activity of alpha-amylase and invertase in the midgut of the cockroach *Diploptera punctata* (Fusé et al., 1999) and the two-spotted cricket *Gryllus bimaculatus* (Woodring et al., 2009), but in *Spodoptera frugiperda* allatostatin inhibited amylase and trypsin activities (Lwalaba et al., 2010). Further, most leucokinins inhibited the release of protease and amylase into the midgut of the lepidopteran larvae *Opisina arenosella*, although one leucokinin with a tyrosine residue (LK VIII) stimulated protease release (Harshini et al., 2002).

Another set of neuropeptides controls general food intake behaviour, including the modulation of chemoreceptors and satiety. For example, sulfakinins (Wei et al., 2000) and corticotropin-releasing factor-like/diuretic hormone (Van Wielendaele et al., 2012) inhibited food intake in the locust *Schistocerca gregaria*, and the latter factor also seems to signal the end of feeding and satiety in *L. migratoria* (Goldsworthy et al., 1999). In addition, neuropeptide F activated specific odorant-receptor neurons and stimulated the odour-driven food search behaviour in *Drosophila melanogaster* (Root et al., 2011), and leucokinin modulated chemosensory functions and food intake in the same species (Al-Anzi et al., 2010; López-Arias et al., 2011).

Recent studies reported that the adipokinetic hormone (AKH) plays an important role in the control of insect gut functions (Kodrík et al., 2012). This neuropeptide is produced by the corpora cardiaca, a neuroendocrine gland near the brain. The main function of AKHs is the control of energy metabolism (Gäde et al., 1997), but they are involved in several accompanying processes at the cellular, organ, and organismal levels (Gäde & Goldsworthy, 2003; Kodrík, 2008; Kodrík et al., 2015). Thus, in the firebug *Pyrrhocoris apterus* the injection of AKH significantly stimulated lipid and protein consumption and enhanced the activity of the midgut digestive enzymes peptidase and alpha-glucosidase (Kodrík et al., 2012), and in the flesh fly *Sarcophaga crassipalpis* Bil et al. (2014) found that AKH treatment enhanced midgut's proteolytic activity. These results are in accordance with the earlier founding of Sellami et al. (2010) that AKH might regulate food consumption in *D. melanogaster* which was recently confirmed by Gáliková et al. (2017) using AKH- and AKH-receptor (AKHR)-deficient flies. Furthermore, in the American cockroach *Periplaneta americana* both cockroach AKHs (Peram-CAH-I and –II; for the structures see Material and Methods section) stimulated midgut amylase activity not only after their injection but also under *in vitro* conditions (Bodláková et al., 2017). Such direct hormonal stimulation requires a relevant receptor and, indeed, the expression of the *AKHR* gene was reported in the midgut and gastric caeca of the cockroach (Wicher et al., 2006; Bodláková et al., 2017). However, the gut *AKHR* gene was also found in other insect species; the list includes *Sarcophaga crassipalpis* (Bil et al., 2016), the aphid *Pseudoregma bambucicola* (Jedličková et al., 2015), the tobacco hornworm *Manduca sexta* (Ziegler et al., 2011), the cockroach *Blattella germanica* (Huang et al., 2012), and the bumblebee *Bombus terrestris* (Jedlička et al., 2016). Relatively low level of AKHR expression (as compared with the other organs) was found also in dipteran representatives the fruit fly *D. melanogaster* (Veenstra et al., 2008), and the mosquito *Aedes aegypti* (Kaufman et al., 2009).

It is interesting that AKH also stimulates food intake through the activation of salivary glands as reported in *P. apterus* (Vinokurov et al., 2014). Pyrrhocorid bugs are adapted to feeding on dry plant seeds and use partial extra-oral digestion (Silva & Terra, 1994), and thus salivary glands play an important role in the digestive process. The AKH treatment significantly increased the transcription of polygalacturonase mRNA and the activity of this enzyme in *P. apterus* salivary glands (Vinokurov et al., 2014). Polygalacturonase has been

described in several heteropteran species (Frati et al., 2006) and it degrades the polygalacturonan present in plant cell walls by hydrolysing its glycosidic bonds. After piercing the dry (linden) seeds *P. apterus* activates the intrinsic enzymatic cocktail within these seeds (amylase, glucosidase, peptidase, lipase), and it has been found that the activity of these enzymes significantly enhanced when AKH-treated individuals fed on the seeds (Vinokurov et al., 2014). However, the mechanism underlying this enhancement is unknown.

Thus, the main goal of the present study was to revise the stimulatory effect of externally applied AKH on the activity of the main digestive enzymes (protease, amylase, and lipase) present in the gut of the cockroach *P. americana*, and to verify if such stimulation is enzyme-, or even species-specific using the *D. melanogaster* mutant producing a non-functional AKH.

Materials and methods

Experimental insects

Periplaneta americana - A colony of the American cockroach used in the present study was kept in 60 l glass fish tanks in a mass cultures and reared at constant temperature of 30 ± 1° C under short-day conditions (12 h L : 12 h D). The cockroaches were supplied with food (stale bread, oat flakes, carrots, apples) and water ad libitum. As there were found no differences in the studied parameters between sexes (data not shown), adults of unknown age were used for the experiments.

Drosophila melanogaster - The fruit flies were maintained in glass vials with standard corn meal/yeast/sucrose/agar diet at 25°C and 12 h L : 12 h D cycles. All assays were carried out on larvae of the 3rd instar, 3-day after the larval hatching. The fly strains used in this study were as followings: (1) the controls: w^{1118} (Bloomington Centre); (2) Akh^{1} mutant with a three bases deletion in the *Akh* gene on the chromosome III resulting in lack of the third amino acid in the AKH octapeptide (Drome-AKH: pGlu-Leu-Thr-Phe-Ser-Pro-Asp-Trp-NH_2 (Schaffer et al., 1990)) and thus in lack of functionality (Sajwan et al., 2015); (3) *EE-Akh* 'rescue' mutant fly which ectopically express *Akh*, prepared as described previously (Sajwan et al., 2015); briefly, the original Akh^{1} mutant was combined with the *Act-Gal4* driver and crossed with *UAS-Akh*. All mutants were backcrossed to w^{1118} for 8 generations (for details see Zemanová et al., 2016).

Hormonal treatment and the gut dissection from cockroach

The used adipokinetic hormones Peram-CAH-I and Peram-CAH-II (Scarborough et al., 1984) were commercially synthesized in the Polypeptide Laboratories, Praha. They are both octapetides that differ in three amino acids - Peram-CAH-I: pGlu-Val-Asn-Phe-Ser-Pro-Asn-Trp-NH_2 and Peram-CAH-II: pGlu-Leu-Thr-Phe-Thr-Pro-Asn-Trp-NH_2. Suitable doses of the hormones (20, 40 and 80 pmol - selected using a dose-response curve (data not shown)) solved in 20% methanol in Ringer saline were injected (in 2 µl) into the cockroach bodies. The controls were injected by the solvent only. Twenty-four hours later the gastric caeca and midgut were dissected from the insects, carefully dried, weighed and stored at -20^{o}C until used for enzyme activity determination (see below). For the selected hormonal doses and the time of hormonal treatment see Kodrík et al. (2012) and Bodláková et al. (2017).

In another series of experiments the effect of the hormones was tested *in vitro*. Here, the dissected gastric caeca and midgut were incubated for 24 hours in 500 µl of Grace's medium (the amount corresponding with estimated body volume) at 30^{o}C in presence of the same hormonal doses solved in 20% methanol in Ringer saline as for *in vivo* assay (see above);

control incubations received the solvent only. In 24 hours the incubation was terminated, the organs moved from the medium, weighted and again stored at -20^{o}C until used.

Processing of the fruit fly body

No hormonal treatment and no gut dissection were performed in the fruit flies; the enzyme activity was determined in the whole body extract.

Enzyme activity determinations

The activities of proteases, amylases and lipases were determined in the gastric caeca and midgut (cockroach) and the whole body (fruit fly). The samples were homogenized (sonicated) in appropriate buffer (see below), centrifuged and the aliquots – 0.005 equiv. for cockroach, and 2 equiv. (protease, amylase) or 0.25 equiv. (lipase) for fruit fly – were tested for enzyme activity.

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

Amylase assay - The assay was performed with 3,5-dinitrosalicylic acid reagent (DNS) according to Bernfeld (1955) as modified by Kodrík et al. (2012). Briefly, 25 µl aliquot sample extract in 100 mM phosphate buffer (Frugoni, 1957) pH 5.7 was mixed with an equal volume of 2% soluble starch made of the same buffer with 40 mM NaCl. The reaction mixture was incubated at 30°C and under constant agitation for 40 min until it was terminated by adding of 200 µl DNS. Then the solution was heated at 100°C for 5 min, cooled, clarified by centrifugation (10 000 g 10 min), and the absorbance was read in supernatant at 550 nm. Enzyme activity was calculated in µmol maltose per mg of fresh organ weight or per the whole body (fruit fly).

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

Statistical analyses

The results were plotted using the graphic software Prism (Graph Pad Software, version 6.0, San Diego, CA, USA). The bar graphs represent mean ± SD, the numbers of replicates (n)

are depicted in the figure legends. The statistical differences were evaluated by one-way ANOVA with Dunnett's post test (Figs. 1-3) or with Tukey's post test (Fig. 4) using the Prism software.

Results

AKH effect on the gastric caeca and midgut enzymatic activity of P. americana

In the first series of experiments, the effects of Peram-CAH-I and -II on protease activity were tested (Fig. 1). Results showed that protease activity in untreated controls was comparable in the gastric caeca and midgut, and that the injection of Peram-CAH-I and -II into the cockroach body significantly increased protease activity in the midgut (about 1.5- to 1.8-fold increase) but not in gastric caeca (Fig. 1A, 1B). Further, no apparent differences were recorded between Peram-CAH-I and -II effects. Similar results were recorded under *in vitro* conditions, when AKHs were applied into the medium in which gastric caeca and midgut were incubated (Fig. 1C, 1D), except for the effect of 20 pmol Peram-CAH-I in gastric caeca (Fig. 1C). Nevertheless, the hormonal stimulation of protease activity in the midgut was more intense *in vitro* (2.2- to 2.9-fold increase) then *in vivo*. The hormonal stimulation of protease activity under *in vitro* conditions suggested a direct hormonal action, not mediated by any other organ or tissue. Interestingly, the highest AKH dose tested (80 pmol) elicited lower stimulatory response than lower doses in all experiments.

Amylase activity in control treatments, which has already been generally characterized in our previous study (Bodláková et al., 2017), was significantly higher in the gastric caeca than in the midgut: 3.9 and 5.8 times higher *in vivo* and *in vitro*, respectively (Fig. 2). These differences were reduced or even removed after AKHs application, because the midgut seems to be more sensitive to the hormonal treatment than the gastric caeca. Indeed, *in vivo*, hormones elicited a 1.6 to 2.2-fold increase of amylase activity in the gastric caeca and a 4.7 to 7.3-fold increase in the midgut (Fig. 2A, 2B); *in vitro* the increase was 1.4-fold in the gastric caeca and from 5.1- to 8.6-fold in the midgut (Fig. 2C, 2D). Similar to that obtained for protease activity, there were no substantial differences between the tested AKHs and the highest stimulatory response was not reached using the highest hormonal dose.

Interesting results were also found for *P. americana* gut lipases (Fig. 3). Unlike amylases, higher lipase activity was recorded in the midgut than in gastric caeca – 2.3- and 1.3-times higher *in vivo* and *in vitro*, respectively (Fig. 3). Similar differences were recorded after the hormonal treatments, because the stimulation of lipase activity was similar for gastric caeca and midgut. Under *in vivo* conditions, lipase activity in the gastric caeca increased by 1.3- to 1.5-fold and by 1.3- to 1.8-fold in the midgut (Fig. 3A, 3B); *in vitro*, lipase activity increased 1.3 to 1.5 fold in gastric caeca and 1.5 to 1.8 fold in midgut (Fig. 3C, 3D). Once again, no significant differences on lipase activity were recorded between the tested hormones and the highest tested AKH doses elicited lower stimulatory responses (although not significant) than lower doses.

AKH effect on D. melanogaster's enzymatic activity

The above-mentioned positive effect of AKHs on the enzymatic activity of *P. americana* gut was confirmed by using another insect species (Fig. 4). Two strains of *D. melanogaster*, one producing deficient, non-functional AKH (Akh^1) and one with rescued AKH production (*EE-Akh*), were employed. The activities of the three monitored enzymes – protease (Fig. 4A), amylase (Fig. 4B), and lipase (Fig. 4C) – were lower in the Akh^1 strain, although lipase

activity was not significantly lower. Furthermore, enzymatic activities in *EE-Akh* flies were similar or even significantly higher than in w^{1118} controls.

Discussion

Activity of digestive enzymes

The digestive system of insects is extraordinary variable and its anatomy, digestive processes, and enzymatic equipment is adapted to the type of food ingested. Two different experimental models representing phylogenetically distant insects, adult *P. americana* and larval *D. melanogaster*, were used in the present study. Despite these main differences, the nutritional quality of their food was similar in our colonies: bread, oat flakes, and vegetables were supplied to cockroaches, and a standard diet containing corn meal, yeast, sucrose, and agar was supplied to fruit flies.

The detailed investigation of *P. americana*'s digestive enzymes conducted in this study revealed that all studied digestive enzymes were active both in the gastric caeca and midgut, although their activities differed between these portions of the intestine: relative amylase activity was higher in gastric caeca than in midgut, relative lipase activity was by contrast higher in midgut than in gastric caeca, and relative protease activity was similar in both. Considerable compartmentalization of enzymatic digestion within *P. americana*'s midgut was also recorded by Tamaki et al. (2015) who found high enzymatic activity in the foregut (crop) (amylase, maltase, trypsin, aminopeptidase) and in the hindgut. Nevertheless, the latter activity was only recorded in gut contents, not in the hindgut tissues, and thus enzymes apparently originated in the midgut and arrived into the hindgut with the chyme. Differentiated enzymatic activity was also observed along the midgut of other insect species. In the firebug *P. apterus*, the highest lipase and alpha-glucosidase activities were recorded in the anterior midgut, while peptidase activity was lowest in this midgut portion and significantly increased in middle and posterior midgut (Kodrík et al., 2012). This was similar to that registered in the pyrrhocorid *Dysdercus cingulatus*, where the highest peptidase activity was located in the posterior midgut (Silva & Terra, 1994). This highest activity level can be explained by the presence of specialized peptidase-producing cells in this portion of the midgut or by the accumulation of enzymes transferred from previous midgut portions; however, the role of symbiotic microorganism cannot be excluded (Haas & König, 1987; Terra & Ferreira, 2005, 2012). In *D. melanogaster*, the midgut is divided into six major anatomical regions (R0 to R5) for practical reasons and to provide a better description of digestive and metabolic functions (Buchon et al., 2013), and, based on bioinformatics predictions (Lemaintre & Miguel-Aliaga, 2013), the *D. melanogaster* genome encodes up to 349 putative digestive enzymes. Expression analyses revealed that amylases were present in regions R2 and R4, while enzymes involved in the digestion of simpler carbohydrates were found in R4 and R5 (Buchon et al., 2013). Nevertheless, reasons for this restricted expression are not fully understood, given that digestive enzymes can easily diffuse in the midgut lumen (Lemaintre & Miguel-Aliaga, 2013).

Hormonal control of the activity of digestive enzymes

It has been known for a long time that AKH activities are not limited only to energy metabolism (Gäde & Goldsworthy, 2003; Gäde et al., 2003; Kodrík, 2008), but just recently AKHs were found to be involved also in the control of food ingestion and gut functions, which fits into the general AKH roles mentioned above. The first indications that AKH might affect gut functions resulted from studies demonstrating the expression of AKHR in the insect gut (*P. americana*, Wicher et al., 2006; *M. sexta*, Ziegler et al., 2011; *B. germanica*, Huang et

al., 2012). However, the first evidence describing AKH's role in digestion was obtained for the firebug *P. apterus*, where the injection of Pyrap-AKH significantly stimulated peptidase and alpha-glucosidase activities in its midgut (Kodrík et al., 2012). The stimulatory effect of AKH on the gut digestive enzymes was also recorded by Bil et al. (2014) in *S. crassipalpis*. These authors found that injection of AKH into decapitated flies or sugar-fed intact flies enhanced midgut proteolytic activity up to the level found in intact flies. However, AKH controls digestive processes also by affecting the gut muscle activities. Stoffolano et al. (2010, 2014) demonstrated that the injection of Phote-HrTH (*Phormia terraenovae* hypertrehalosemic hormone) into the fly *Phormia regina* affected food carbohydrates transfer from the crop into the midgut. On the other hand, Konuma et al. (2012) found that the knockdown of AKHR by RNA interference in *Gryllus bimaculatus* increased feeding frequency, which indicated that the feeding control function of AKH might be species-specific.

In a recent study (Bodláková et al., 2017), we provided a detailed biochemical characterization of alpha-amylase in *P. americana* midgut, which included the following characteristics: optimal pH, 5.7; optimal temperature 38.4°C; Michaelis-Menten constant (K_m), 2.54 mg starch/ml; and maximum reaction velocity (V_{max}), 0.185 μmol maltose/ml/min. However, and most importantly, we found that the injection of AKHs into this cockroach body significantly stimulated amylase activity in gastric caeca and in the midgut, which was confirmed by *in vitro* experiments applying tested AKHs to the medium where gut portions were incubated. This suggested direct hormonal stimulation of amylase activity not mediated by other organs or inter-organ cascades. Such functional cascades are known and/or are supposed for some indirect AKH activities as e.g. stimulation of locomotory activity (Socha et al., 1999) or inhibition of egg maturation (Lorenz, 2003). In the present study, the stimulatory effect of AKH on *P. americana* amylases was corroborated, and it was demonstrated that AKHs also affected other digestive enzymes in the cockroach gut, namely proteases and lipases. Results of *in vitro* experiments demonstrated the same direct hormonal effects on protease and lipase, as suggested for amylase by Bodláková et al. (2017).

The hormonal stimulation of enzyme activities was not uniform along the *P. americana* midgut, as AKHs affected enzymes selectively, and, in some cases, unequally in gastric caeca and midgut; in addition, no substantial differences were recorded between the stimulation of both *P. americana* AKHs. Interestingly, protease activity was only hormonally stimulated in the midgut, with the exception of *in vitro* stimulation using 20 pmol Peram-CAH-I. However, this selective expression might not be linked to AKHR only, because the relative expression of the *AKHR* gene was about 20 times higher in the gastric caeca than in the midgut (Bodláková et al., 2017). Additionally, amylase and lipase activities in the gastric caeca were stimulated by the AKH treatment (present study). Thus, cells specialized on the production of proteases and having the AKHR might be present in the midgut; however, without any further analyses this remains just a hypothesis. Further, as mentioned above, amylase activity was higher in the gastric caeca than that in the midgut of intact cockroaches, but, because the overall rate of hormonal stimulation was more intensive in the midgut, the difference in amylase activity was largely eliminated in treated cockroaches. On the other hand, lipase activity was more intensive in the midgut than in the gastric caeca of untreated controls, and this difference was maintained after the hormonal treatment. Nutrients digestion seems, therefore, to be compartmentalized to some extent in the cockroach gut, and AKHs seem to stimulate it by a complicated mechanism that is not clear at present.

It is interesting to note that the stimulatory effect of the maximal hormonal dose (80 pmol) on the enzyme activities of the midgut was at least partially lower than the effect of lower doses in all experimental sets. This phenomenon, i.e. the maximal response is elicited by an optimal dose and lower or higher doses are less efficient, is a typical feature of AKHs and it has been recorded in other assays. For example, the maximal effect of Pyrap-AKH on lipid mobilization in *P. apterus* body was obtained with 10 pmol of hormone (Kodrík et al., 2000), and the maximal stimulatory effect on the locomotor activity of the same species required a 40 pmol injection (Kodrík et al., 2002); other tested doses were less effective in both experiments. We have no satisfactory explanation for this phenomenon, nevertheless, one can speculate that higher AKH doses might affect AKH vs. receptor interactions. However, any direct evidence is missing.

The stimulatory effect of AKHs on enzymatic activity was also studied in the present paper using *D. melanogaster*. A selection of this model was not random, because it allowed us to verify the effect of classical AKH application obtained on the cockroaches with the opposite effect of absence of functional AKH obtained by the gene manipulation. For those experiments whole larval bodies were used for enzyme extraction, thus, not only gut enzymes were evaluated, although it is likely that those enzymes accounted for the majority of the results. A significant decrease of amylase and protease activities was recorded in the Akh^{1} strain producing non-functional Drome-AKH, while the reduction of lipase activity was less intense in these larvae. This situation is in accordance with the findings of Sajwan et al. (2015), who recorded significantly lower levels of total carbohydrates and trehalose in Akh^{1} larval bodies. A lower effect of AKH on lipase activity might be explained by a lower level of lipids in the food and thus lower importance of lipases in *Drosophila* digestion; if there are some other metabolic reasons remains to be speculation. The interesting role of AKH in *D. melanogaster* has been recently described by Gáliková et al. (2017), who reported Drome-AKH increased food intake in these flies, despite its anti-obesity function. Both effects are likely to be controlled by AKH independently, but the mechanism underlying this control might be complicated because Drome-AKH also regulates the expression of orexigenic (= appetite stimulating) factors and metabolic hormones, including corazonin and insulin-like peptides (Gáliková et. al., 2017).

Not only AKHs regulate insect gut functions and several studies and review articles (e.g., Audsley & Weaver, 2009; Spit et al., 2012; Schoofs et al., 2017) have described the involvement of other neuropeptides. Many of these neuropeptides were detected or predicted by molecular genome sequencing, transcriptomic, proteomic, or peptidomic analyses, but the knowledge on their functions are often lagging behind. Nevertheless, they are mostly responsible for the control of gut motility, allowing food/chyme passage through the alimentary system, or for the synthesis and release of digestive enzymes. As mentioned also in Introduction the neurohormonal effect on activity of digestive enzymes is sometimes opposed in different species. The stimulatory effect of AKH on digestive enzymes has been proven in a few insect species so far: *P. apterus* (Kodrík et al., 2012), *S. crassipalpis* (Bil et al., 2014), *P. americana* (Bodláková et al., 2017; this study) and *D. melanogaster* (this study). Nevertheless, the inhibitory effect of AKH has never been recorded.

In conclusion, the present study demonstrated AKH stimulation of proteases, amylases, and lipases in the gastric caeca and midgut of the cockroach *P. americana*. This stimulation was not uniform, but it was recorded in both *in vivo* and *in vitro* experiments, suggesting a direct stimulation. In agreement, enzymatic activities in *D. melanogaster* larvae producing

non-functional AKH were lower than in the larvae with ectopically expressed *Akh* gene, where enzymatic activity reached or even exceeded that of controls.

Acknowledgements

This study was supported by grant No. 17-03253S (DK) from the Czech Science Foundation, and by projects RVO 60077344 of the Institute of Entomology. The authors thank Mrs. H. Štěrbová and Mrs. A. Žaloudíková for their technical assistance. The English grammar and stylistics were checked by the Editage Author Services.

References

Al-Anzi, B., Armand, E., Nagamei, P., Olszewski, M., Sapin, V., Waters, C., Zinn, K., Wamyn, R.J. & Benzer S. (2010) The leucokinin pathway and its neurons regulate meal size in *Drosophila*. *Current Biology,* **20**, 969-978.

Audsley, N. & Weaver, R.J. (2009) Neuropeptides associated with the regulation of feeding in insects. *General and Comparative Endocrinology*, **162**, 93-104.

Bernfeld, P. (1955) Amylases, α and β. *Methods in Enzymology*, (ed. by Colowick, S.P. and Kaplan, N.O.), Vol. 1, pp. 149-158, Academic Press, New York.

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. General and Comparative Endocrinology,* **208**, 49-56.

Bil, M., Timmermans, I., Verlinden, H. & Huybrechts, R. (2016) Characterization of the adipokinetic hormone receptor of the anautogenous flesh fly, *Sarcophaga crassipalpis*. *J. Insect Physiol*., **89**, 52-59.

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

Buchon, N., Osman, D., David, F.P.A., Yu Fang, H., Boquete, J.P., Deplancke, B. & Lemaitre, B. (2013) Morphological and molecular characterization of adult midgut compartmentalization in *Drosophila*. *Cell Reports*, **3**, 1725-1738.

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. *Archives of Insect Biochemistry and Physiology*, **48**, 206–216.

Frati, F., Galletti, R., De Lorenzo, G., Salerno, G. & Conti, E. (2006) Activity of endo-polygalacturonases in mirid bugs (Heteroptera: Miridae) and their inhibition by plant cell wall proteins (PGIPs). *European Journal of Entomology,* **103**, 515-522.

Frugoni, J.A.C. (1957) Tampone universale di Britton e Robinson a forza ionica costante. *Gazzetta Chimica Italiana*, **87**, 403-407.

Fusé, Zhang, J.R., Partridge, E., Nachman, R.J., Orchard, I., Bendena, W.G. & Tobe, S.S. (1999) Effects of an allatostatin and a myosuppressin on midgut carbohydrate enzyme activity in the cockroach *Diploptera punctata*. Peptides, **20**, 1285-1293.

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. & Spring, J.H. (1997) Hormonal regulation in insects: facts, gaps and future directions. *Physiological Review*, **77**, 963-1032.

Gäde, G., Auerswald, L., Šimek, P., Marco, H.G. & Kodrík D. (2003) Red pigment concentrating hormone is not limited to crustaceans. *Biochemical and Biophysical Research Communication*, **309**, 967-973.

Gáliková, M., Klepsatel, P., Xu, Y. & Kühnlein R.P. (2017) The obesity-related adipokinetic hormone controls feeding and expression of neuropeptide regulators of *Drosophila* metabolism. *European Journal of Lipid Science and Technology*, **119**, 1600138.

Goldsworthy, G.J., Owusu, M., Ross, K.T.A., Simmonds, M.S.J., Coast, G.M. & Blaney, W.M. (1999) *Locusta* CRF related diuretic peptide is a factor mediating satiety in locusts. *Recent Developments in Comparative Endocrinology and Neurobiology* (ed. by Roubos, E.W., Wendelaar Bonga, S.E., Vaudry, H. and De Loof, A.), pp. 231-234, Shaker Publishing BV, Maastricht.

Haas, F. & König, H. (1987) Characterization of an anaerobic symbiont and the associated aerobic bacterial flora of *Pyrrhocoris apterus* (Heteroptera: Pyrrhocoridae). *FEMS Microbiology Letters*, **45**, 99-106.

Harshini, S., Nachman, R.J. & Sreekumar, S. (2002) Inhibition of digestive enzyme release by neuropeptides in larvae of *Opisina arenosella* (Lepidoptera : Cryptophasidae). *Comparative Biochemistry and Physiology B*, **132**, 353-358.

Huang, J.H., Bellés, X. & Lee, H.J. (2012) Functional characterization of hypertrehalosaemic hormone receptor in relation to haemolymph trehalose and to oxidative stress in the cockroach, *Blattella germanica. Frontiers in Endocrinology*, **2**, Article 114.

Jedlička, P., Ernst, U.R., Votavová, A., Hanus, R. & Valterová, I. (2016) Gene expression dynamics in major endocrine regulatory pathways along the transition from solitary to social life in a bumblebee, *Bombus terrestris. Frontiers in Physiology*, **7**, article 574.

Jedličková, V., Jedlička, P. & Lee, H.J. (2015) Characterization and expression analysis of adipokinetic hormone and its receptor in eusocial aphid *Pseudoregma bambucicola*. *General and Comparative Endocrinology*, **223**, 38-46.

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

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

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

Kodrík, D., Bednářová, A., Zemanová, M. & Krishnan, N. (2015) Hormonal regulation of response to oxidative stress in insects - an update. *International Journal of Molecular Science*, **16**, 25788-25816.

Kodrík, D., Vinokurov, K., Tomčala, A. & Socha, R. (2012) The effect of adipokinetic hormone on midgut characteristics in *Pyrrhocoris apterus* L. (Heteroptera). *Journal of Insect Physiolology*, **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 Biochemistry and Molecular Biology*, **30**, 489-498.

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

Lemaitre, B. & Miguel-Aliaga, I. (2013) The digestive tract od *Drosophila melanogaster*. *Annual Review of Genetics*, **47**, 377-404.

Lopez-Arias, B., Dorado, B. & Herrero, P. (2011) Blockade of the release of the neuropeptide leucokinin to determine its possible functions in fly behavior: chemoreception assays. *Peptides*, **32**, 545-552.

Lorenz, M.W. (2003) Adipokinetic hormone inhibits the formation of energy stores and egg production in the cricket *Gryllus bimaculatus. Comparative Biochemistry and Physiology Part B*, **136**, 197-206.

Lwalaba, D., Hoffmann, K.H. & Woodring, J. (2010) Control of the release of digestive enzymes in the larvae of the fall armyworm, *Spodoptera frugiperda. Archives of Insect Biochemistry and Physiol*ogy, **73**, 14-29.

Nachman, R.J., Holman, G.M., Haddon, W.F. & Ling, N. (1986a) Leucosulfakinin, a sulfated insect neuropeptide with homology to gastrin and cholecystokinin. *Science*, **234**, 71–73.

Nachman, R.J., Holman, G.M., Cook, B.J., Haddon, W.F. & Ling, N. (1986b) Leucosulfakinin II, a blocked sulfated insect neuropeptide with homology to cholecystokinin and gastrin. *Biochemical and Biophysical Research Communication*, **140**, 357–364.

Roberts, I.M. (1985) Hydrolysis of 4-methylumbelliferyl butyrate: a convenient and sensitive fluorescent assay for lipase activity. *Lipids*, **20**, 243-247.

Root, C.M., Ko, K.I., Jafari, A. & Wang J.W. (2011) Presynaptic facilitation by neuropeptide signaling mediates odor-driven food search. *Cell*, **145**, 133-144.

Sajwan, S., Sidorov, R., Stašková, T., Žaloudíková, A., Takasu, J., Kodrík, D. & Žurovec, M. (2015) Targeted mutagenesis and functional analysis of adipokinetic hormone-encoding gene in *Drosophila*. *Insect Biochemistry and Molecular Biology,* **61**, 79-86.

Scarborough, R.M., Jamieson, G.C., Kalish, F., Kramer, J.S., McEnroe, G.A., Miller, C.A. & Schooley, D. (1984) Isolation and primary structure of two peptides with cardioacceleratory and hyperglycemic activity from the corpora cardiaca of *Periplaneta americana*. *Proceedings of the National Academy of Science USA*, **81**, 5575–5579.

Schaffer, M.H., Noyes, B.E., Slaughter, C.A., Thorne, G.C. & Gaskell, S.J. (1990) The fruitfly Drosophila melanogaster contains a novel charged adipokinetic-hormone-family peptide. *Biochemical Journal*, **269**, 315-320.

Schoofs, L., De Loof, A. & Van Hiel, M.B. (2017) Neuropeptides as regulators of behaviour in insects. *Annual Review of Entomology,* **62**, 35-52.

Sellami, A., Isabel, G. & Veenstra J.A. (2010) Expression of the mu opioid receptor in Drosophila and its effects on trehalose and glycogen when expressed by the AKH neuroendocrine cells, *Peptides*, **31**, 1383-1389

Silva, C.P. & Terra, W.R. (1994) Digestive and absorptive sites along the midgut of the cotton seed cucker bug *Dysdercus peruvianus* (Pyrrhocoridae). *Insect Biochemistry and Molecular Biology*, **24**, 493–505.

Socha R., Kodrík D. & Zemek R. (1999) Adipokinetic hormone stimulates insect locomotor activity. *Naturwissenschaften* **88**, 85-86

Spit, J., Badisco, L., Verlinden, H., Van Wielendaele, P., Zels, S., Dillen, S. & Broeck, J.V. (2012) Peptidergic control of food intake and digestion in insects. *Canadian Journal of Zoology*, **90**, 489-506.

Stoffolano, Jr., J.G, Guerra, L., Carcupino, M., Gambellini, G. & Faustu, A.M. (2010) The diverticulated crop of adult *Phormia regina*. *Arthropod Structure & Development*, **39**, 261-260.

Stoffolano, Jr., J.G., Croke, K., Chambers, J., Gäde, G., Solari, P. & Liscia, A. (2014) Role of Phote-HrTH (*Phormia terraenovae* hypertrehalosemic hormone) in modulating the supercontractile muscles of the crop of adult *Phormia regina* Meigen. *Journal of Insect Physiology*, **71**, 147-155.

Tamaki, F.K., Pimentel, A.C., Dias, A.B., Cardoso, C., Ribeiro, A.F., Ferreira, C. & Terra, W.R. (2014) Physiology of digestion and the molecular characterization of the major digestive enzymes from *Periplaneta americana*. *Journal of Insect Physiology,* **70**, 22-35.

Terra, W.R. & Fereira, C. (2005) Biochemistry of digestion. Comprehensive Molecular Insect Science (eds. by Gilbert, L.I., Iatrou, K. and Gill, S.S.), Vol. 4, pp.171-224, Pergamom Press, Elsevier, Oxford.

Terra, W.R. & Fereira, C. (2012) Biochemistry and molecular biology of digestion. *Insect Molecular Biology and Biochemistry*, (ed. by Gilbert, L.I.), pp. 365-417, Academic Press, Elsevier, London.

Van Wielendaele, P., Dillen, S., Marchal, E., Badisco, L. & Vanden Broeck, J. (2012) CRF-like diuretic hormone negatively affects both feeding and reproduction in the desert locust, *Schistocerca gregaria*. *PloS ONE*, **7**, e31425

Veenstra, J.A., Agricola, H.J. & Sellami., A. (2008) Regulatory peptides in the fruit fly midgut. *Cell and Tissue Research*, **334**, 499-516.

Vinokurov, K., Bednářová, A., Tomčala, A., Stašková, T., Krishnan, N. & Kodrík, D. (2014) Role of adipokinetic hormone in stimulation of salivary gland activities: the fire bug *Pyrrhocoris apterus* L. (Heteroptera) as a model species. *Journal of Insect Physiology*, **60**, 58-67.

Wei, Z., Baggerman, G., Nachman, R.J., Goldsworthy, G., Verhaert, P., De Loof, A. & Schoofs, L. (2000) Sulfakinins reduce food intake in the desert locust, *Schistocerca gregaria*. *Journal of Insect Physiology*, **46**, 1259–1265.

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. *Journal of Neurophysiology*, **95**, 2314-2325.

Woodring, J., Diersch, S., Lwalaba, D., Hoffmann, K.H. & Meyering-Vos, M. (2009) Control of the release of digestive enzymes in the caeca of the cricket *Gryllus bimaculatus. Physiological Entomology*, **34**, 144-151.

Zels, S., Dillen, S., Crabbe, K., Spit, J., Nachman, R.J. & Broeck, J.V. (2015) Sulfakinin is an important regulator of digestive processes in the migratory locust, *Locusta migratoria*. *Insect Biochemistry and Molecular Biology,* **61**, 8-16.

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

Ziegler, R., Isoe, J., Moore, W., Riehle, M.A. & Wells, M.A. (2011) The putative AKH receptor of the tobacco hornworm, *Manduca sexta*, and its expression. *Journal of Insect Science*, 11, article 40.

Figures:

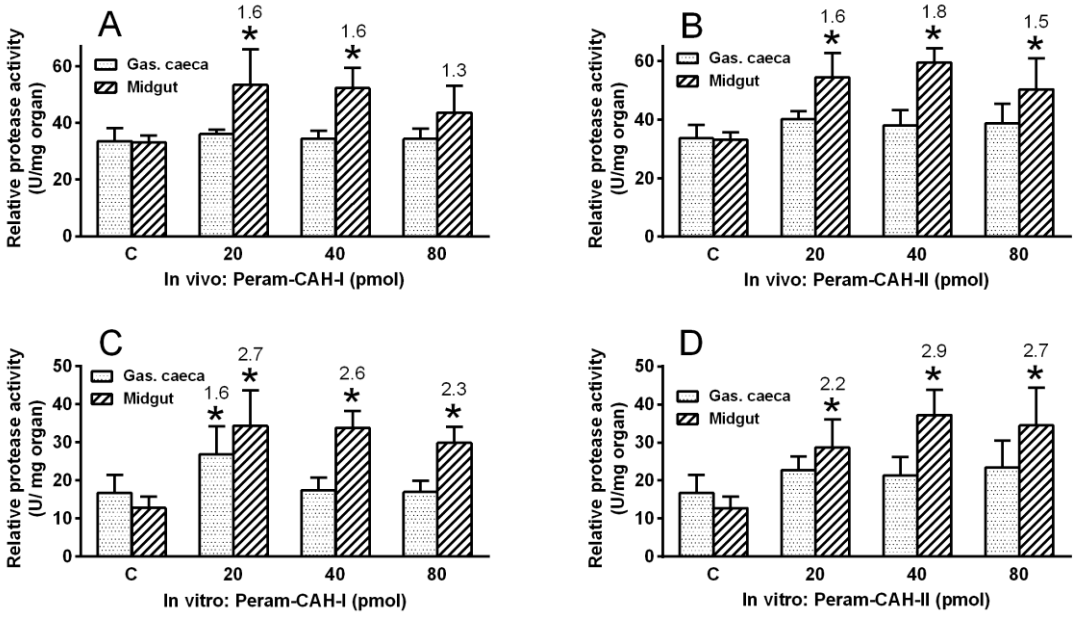

Fig. 1. Levels of protease activity in *P. americana* gastric caeca and midgut determined 24 hours after the injection of Peram-CAH-I (A) or Peram-CAH-II (B), or after the incubation in medium (500 µl) in presence of Peram-CAH-I (C) or Peram-CAH-II (D). Control individuals were injected by 20% methanol in Ringer saline only (2 µl; *in vivo* test), control gastric caeca or midguts were incubated in presence of the same solution (2 µl; *in vitro* test). The numbers above the columns represent fold-difference of gastric caeca/midgut protease activity after the hormonal treatment as compared with corresponding controls. Statistically significant differences between the columns (experimental vs. control) were evaluated using one-way ANOVA with Dunnett's post test; the differences at the 5% level are indicated by asterisks; n=5-6.

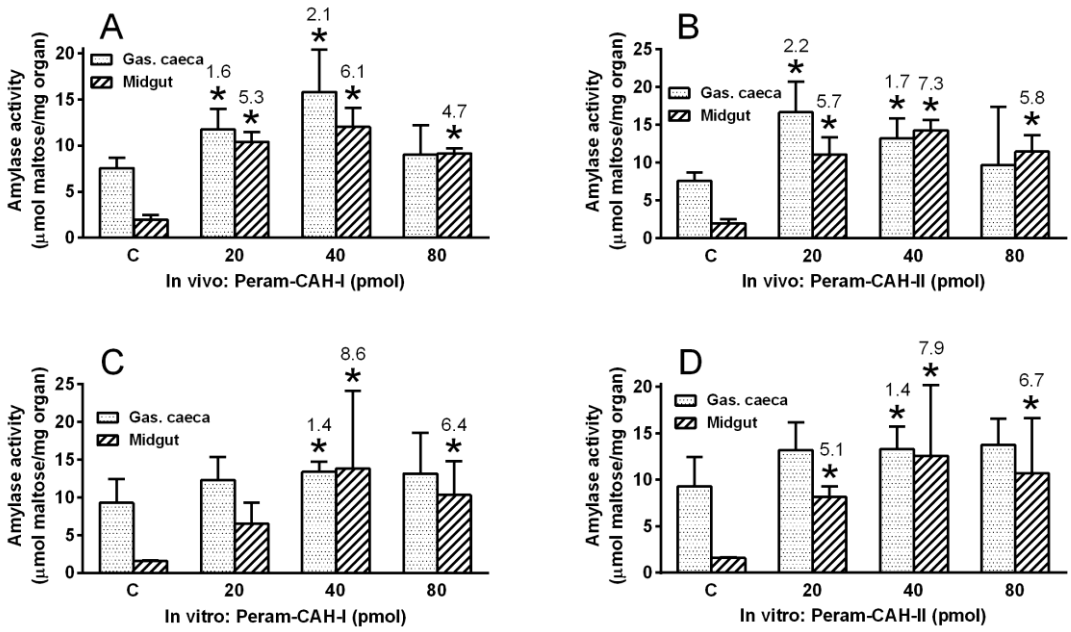

Fig. 2. Levels of amylase activity in *P. americana* gastric caeca and midgut determined 24 hours after the injection of Peram-CAH-I (A) or Peram-CAH-II (B), or after the incubation in medium (500 µl) in presence of Peram-CAH-I (C) or Peram-CAH-II (D). Control individuals were injected by 20% methanol in Ringer saline only (2 µl; *in vivo* test), control gastric caeca or midguts were incubated in presence of the same solution (2 µl; *in vitro* test). The numbers above the columns represent fold-difference of gastric caeca/midgut amylase activity after the hormonal treatment as compared with corresponding controls. Statistically significant differences between the columns (experimental vs. control) were evaluated using one-way ANOVA with Dunnett's post test; the differences at the 5% level are indicated by asterisks; n=5-9. Adapted according to Bodláková et al., 2017.

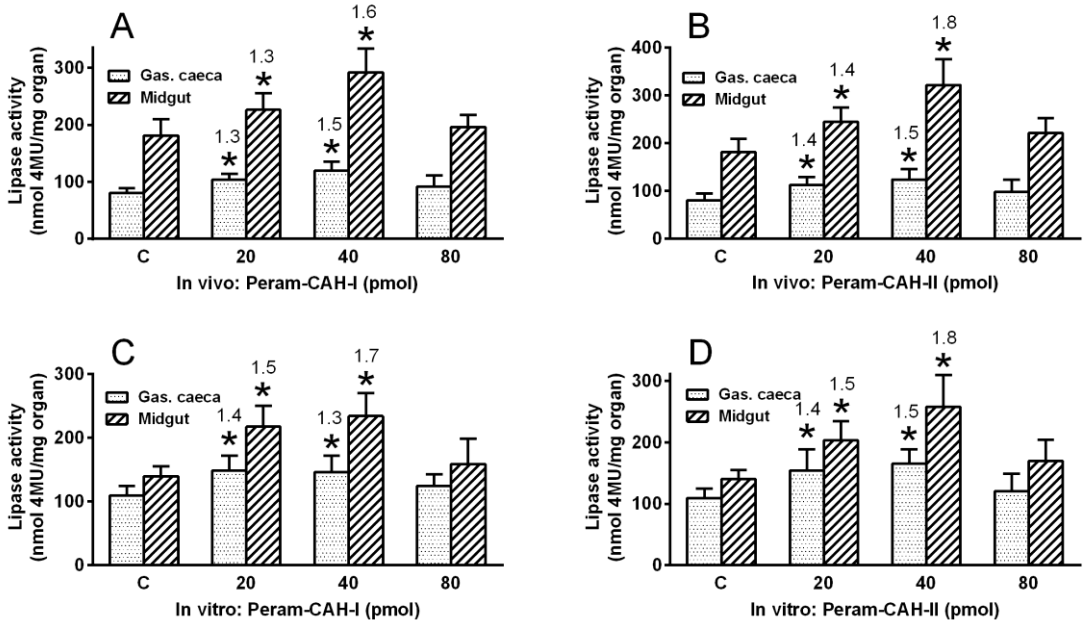

Fig. 3. Levels of lipase activity in *P. americana* gastric caeca and midgut determined 24 hours after the injection of Peram-CAH-I (A) or Peram-CAH-II (B), or after the incubation in medium (500 µl) in presence of Peram-CAH-I (C) or Peram-CAH-II (D). Control individuals were injected by 20% methanol in Ringer saline only (2 µl; *in vivo* test), control gastric caeca or midguts were incubated in presence of the same solution (2 µl; *in vitro* test). The numbers above the columns represent fold-difference of gastric caeca/midgut lipase activity after the hormonal treatment as compared with corresponding controls. Statistically significant differences between the columns (experimental vs. control) were evaluated using one-way ANOVA with Dunnett's post test; the differences at the 5% level are indicated by asterisks; n=5-6.

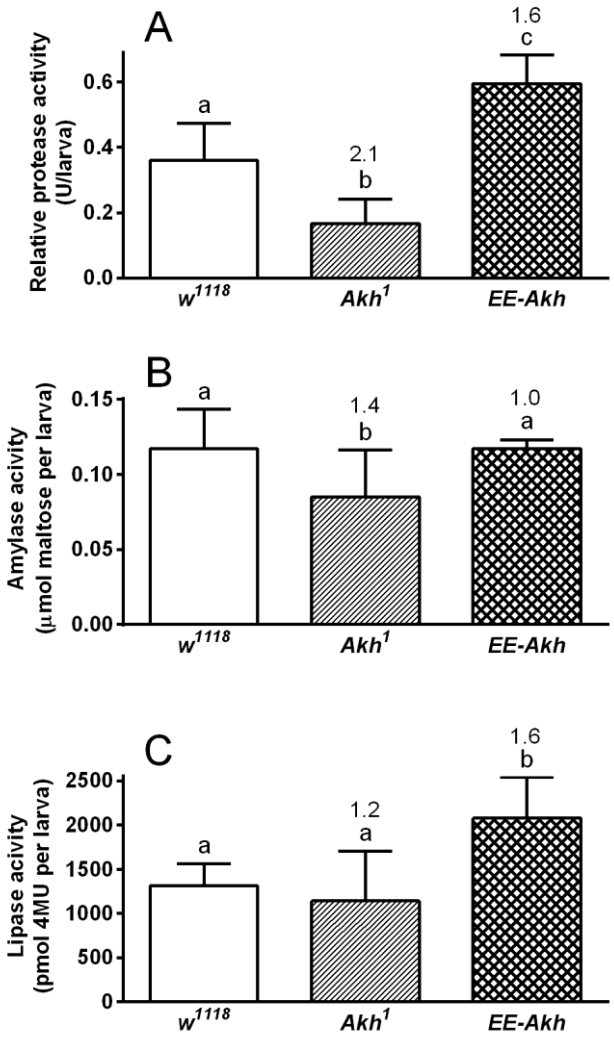

Fig. 4. Levels of protease (A), amylase (B) and lipase (C) activity in the body of various mutants of *D. melanogaster* last instar larvae. The numbers above the columns represent fold-difference of enzyme activity compared with the w^{1118} control. Statistically significant differences among the columns were evaluated using one-way ANOVA with Tukey's post test; the differences at the 5% level are indicated by different letters; n=8.