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ROLE OF ADIPOKINETIC HORMONE PERAM-CAH-II IN INSECT REPRODUCTION

Bachelor thesis

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Biological Chemistry

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Annotation

The aim of this thesis was to investigate the role of adipokinetic hormone Peram-CAH-II in the insect reproduction by analysing vitellogenin levels in haemolymph of *Pyrrhocoris apterus* after hormonal treatment.

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

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Content

1. Introduction

1.1 Insect reproductive system

Reproduction is the function of the sexual reproductive system. In insects, most species reproduce bisexually; egg produced by the female will not usually develop unless fertilized by the spermatozoa produced by the male. Nevertheless, there are some species that reproduce by parthenogenesis, which is characterized by development of unfertilized egg. Except of few species, only one sex is represented in one individual. Adult insects are seldom sexually mature immediately after emerging from the preadult stage. Most insects are oviparous, they lay eggs, but the egg-laying habits differ greatly from species to species.

1.1.1 Male reproductive system

The main function of male's reproductive system is to produce and store spermatozoa until they are brought together with female eggs. The male's reproductive system (Figure 1) contains a pair of testes, usually located near the back of the abdomen. Each testis is subdivided into hundreds functional units, follicles, where sperms are actually produced. The upper portion of the follicles contains primary germ cells, spermatogonia. These divide repeatedly by mitosis to form spermatocytes, which move to the base of the follicle. After the multiplication stage, spermatocytes undergo meiosis, yielding haploid spermatids. This is followed by a transformation period in which the round cells spermatids mature into slender flagellate spermatozoa. Mature sperms leave testes through short ducts called vas efferens and are collected in storage chambers, seminal vesicles. Similar ducts, vas deferens, lead away seminal vesicles to form single ductus ejaculatorius that transport spermatozoa to the male copulatory organ called aedeagus (Gullan and Cranston, 2010).

Single or paired accessory glands are associated with the male's reproductive system. These secretory organs have two main functions. Firstly, they produce seminal fluid that nourishes mature sperms in male's genital tract. The fluid also helps mating and can contain compounds affecting female's behaviour after copulation. Secondly, it manufactures spermatophores, which surround, protect and sustain sperms as they are delivered to the female's body.

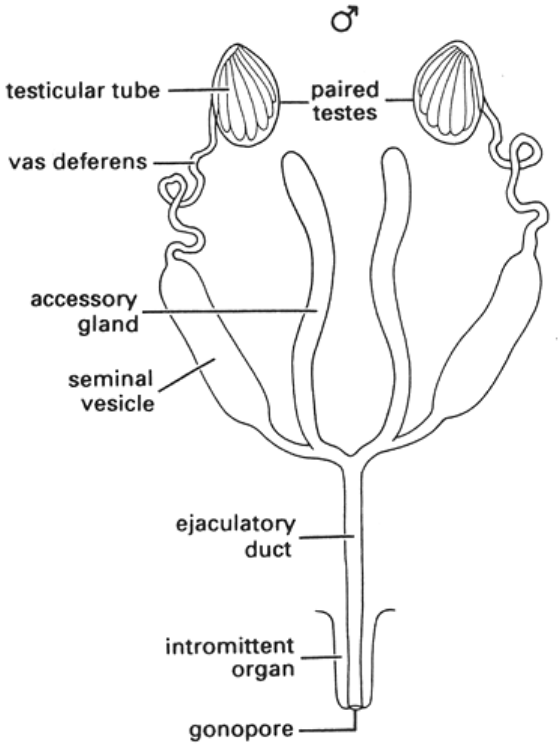

Figure 1: Male reproductive system. After Gullan and Cranston (2010).

1.1.2 Female reproductive system

The main function of female's reproductive system is to produce and protect eggs as well as to store sperms till the eggs are ready to be fertilized. Female's reproductive system (Figure 2) consists of ovaries that are made of ovarioles in which eggs are produced, spermatheca in which sperms are stored and duct arrangement through which eggs are discharged outside the body. Ovarioles are typically aligned parallel to each other and at the apex of each ovariole, there is germarium containing a group of germ cells called oogonia that differentiate to produce oocytes, nutritive cells and follicular epithelial cells. The oocytes grow in size from accumulating yolk mostly called vitellin, composed of proteins, lipids, glycides and other components transported in most insect species (except Diptera) from the fat body as vitellogenins. A middle part of ovariole, where the vitellogenesis is the most intensive is called vitellarium. Afterwards, the follicular epithelium secretes the chorion and the vitelline membrane and then together with the nutritive cell disintegrates. The bottom of an ovary forms a calyx through which mature oocytes pass into lateral oviduct. Near the midline of the body, these lateral oviducts join to form a common oviduct

which opens into an egg-holding genital chamber called the bursa copulatrix (Gullan and Cranston, 2010).

Two structures are connected to the wall of the oviduct. One is spermatheca, which is a single bulbous organ used as a storage chamber for sperms. Near the duct connecting spermatheca with the oviduct, a spermathecal gland is attached. It produces enzymes for digesting the protein coat of the spermatophore and nutrients. The other structure is paired accessory glands, which secret adhesive protein-rich materials that surround the egg. In some species, these glands develop other function, for instance in Hymenoptera they serve as poisonous glands (Gullan and Cranston, 2010).

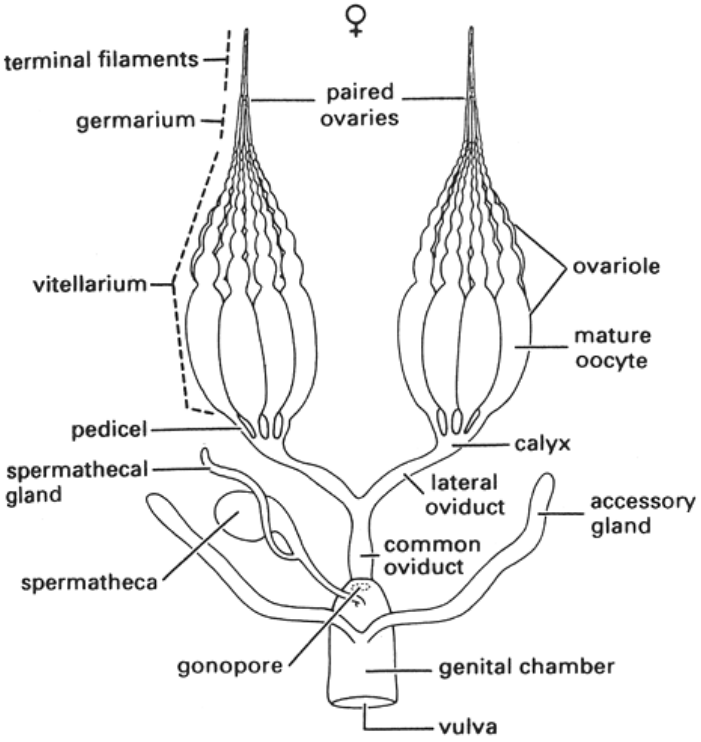

Figure 2: Female reproductive system. After Gullan and Cranston (2010).

1.1.2.1 Vitellogenesis

Vitellogenesis is one of the most important processes occurring in maturing oocytes. It is a process of yolk (vitellus) formation via nutrients absorption by the growing oocyte. Accumulation of nutrients involves massive synthesis of vitellogenin protein (Vg) and its deposition into oocyte. Vitellin (Vn) is a major protein found in yolk, it is derived from vitelogenins utilized as nourishment during embryogenesis. Vitellogenins are phospho-lipo-glyco-proteins existing as several different subunits whose expression shows development,

sexual, hormonal and tissue specificity (Swevers *et al.*, 2005). Vitellogenins are synthetized in trophocytes (adipocytes) of the fat body together with other yolk protein precursors, additionally some species (Diptera) are able to synthetized them directly in ovaries. Three distinctive phases have been identified in trophocytes during each oogenesis cycle: 1. the previtellogenic phase, in which cells have contracted nucleus, more glycogen and large lipid inclusions; 2. the vitellogenic phase, during which cells accommodate abundant rough endoplasmic reticulum and Golgi apparatus, large nucleus and multilobed nucleus; this phase is characterized by extremely intensive synthesis of protein core of Vg; and 3. termination phase, characterized by presence of abundant lysosomes. Vitellogenesis in insects is controlled by several hormones (Figure 3), however a principal role is played by juvenile hormone and ecdysone (Kunkel and Nordin, 1985).

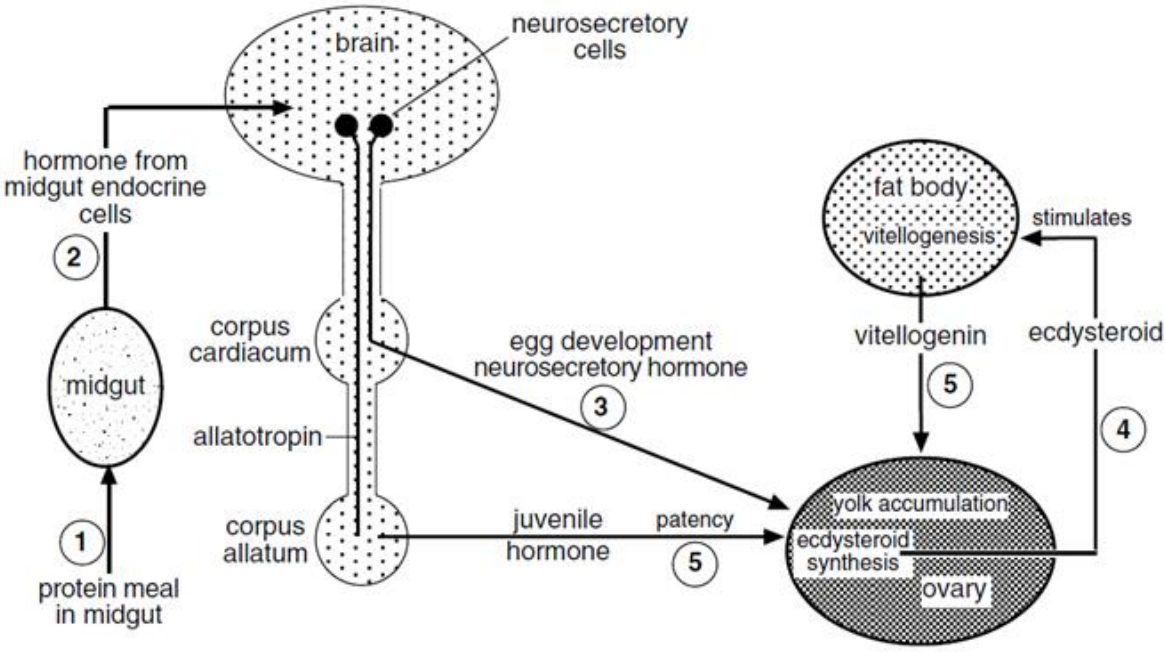

Figure 3: Hormonal control of vitellogenin synthesis. After Chapman (1998).

1.2 Insect endocrine system

Multicellular organisms had to develop a communication system between cells, tissues and organs to coordinate response to stimuli and to regulate biochemical and physiological processes. This need gave rise to endocrine and nervous systems. In general, the nervous system responds rapidly to short-term changes, while the endocrine system brings about longer-term adaptations by sending out chemical messengers called hormones into the blood stream.

Insect endocrine system is one of the most complex systems in invertebrates. There are six main categories of hormone-producing cells in insect's body: neurosecretory cells in brain and other ganglia, retrocerebral complex (corpora cardiaca – corpora allata), prothoracic glands, gut endocrinal cells, epitracheal cells and gonads. Hormones play a crucial control role in insect biology. They regulate moulting, diapause, reproduction, metabolic activities and general body functions, behaviour, pre-progammated cell death as well as they determine the form at metamorphosis and effect polymorphism. Insect hormones are divided into 3 groups: ecdysteroids, juvenile hormones and large group of peptide neurohormones (Gäde *et al.*, 1997; Gullan and Cranston, 2010).

1.2.1 Ecdysteroids

Ecdysteroids are known as moulting hormones, but they also control many other biological functions with relation to moulting of embryos, larva, nymphs and adult reproduction. They are primarily synthesized in prothoracic glands from cholesterol (or phytosterols in herbivorous insects), but can be produced also by other organs including gonads. They act as steroid hormones via intracellular receptors activating synthesis of new enzymes on DNA level. Ecdysteroids together with juvenile hormones are the main metamorphosis hormones. Ecdysteroids control moulting and metamorphosis during embryogenesis and larval development (Ishaaya *et al.*, 2007). In general, concentration of ecdysteroids in haemolymph increases before apolysis of old cuticle, reaches maximum during or shortly after apolysis and then decreases to low or undetected level after ecdysis. In many insect species, ecdysteroids (mainly ecdysone) are also produced in ovaries. Ovarial ecdysteroids are responsible for oocyte maturation by stimulating meiosis, oocyte release from germarium and oviposition. Ecdysteroids are deposited as conjugates into maturing eggs from which they are released during moulting of embryos. In some species, mostly Diptera, ecdysone which is converted into 20-OH-ecdysone in fat body regulates synthesis of vitellogenin (yolk protein) and its secretion into haemolymph. In males, ecdysteroids affect spermatogenesis (Saunders *et al.*, 2002).

1.2.2 Juvenile hormones

Juvenile hormones (JHs) are acyclic sesquiterpenoids that regulate almost all aspects of insect life such as embryogenesis, metamorphosis, vitellogenin synthesis, gonadal development, behaviour, diapause, caste determination, various polyphenisms (Nijhout, 1994) and metabolism. JHs are produced in corpora allata, retrocerebral paired or unpaired endocrine glands of ectodermal origin (Wigglesworth, 1970). Newly synthesized juvenile hormones are released into haemolymph and transported by juvenile hormone binding protein (also known as juvenile hormone carrier protein). JHs production is controlled by allatotropins and allatostatins (Gäde, 1997). Allatotropins stimulate and allatostatins inhibit JH synthesis in corpora allata. Mechanism of JH action is based on induction of specific transcription. As in the case of ecdysteroids, the JH signal transduction is mediated via intracellular receptors (Tomaschko, 1999). Juvenile hormone is one of the main hormones controlling metamorphosis. It keeps individuals in larval (juvenile) stage by preventing metamorphosis. In absence of JH larval-pupal changes start. The small release of JH (in Holometabola) cause stop of metamorphosis at pupa state, and the development does not continue directly to imago. In adulthood, JH has an opposite effect, its mode of action is a classic example of hormonally stimulated gene expression. Juvenile hormone controls production of eggs by regulating production of vitellogenins, their transport and entrance to oocytes (Chapman, 1998). In males, JH stimulates activity of accessory gland and production of pheromones (Socha et al, 2005). In social insect, juvenile hormones together with ecdysteroids and neurohormones affect caste determination. In general, dominant reproductive individuals (queens) have higher JH titre with the exception of termites, where soldier differentiation requires a greater amount of JH than normal termites (Ross, 1982). Juvenile hormones have effect on phase polymorphism and control colour change (for example in male locust Schistocerca). In several species of locusts, JH is responsible for a change from solitary to gregarious migratory individual (Ross, 1982). Diapause is primary induced by a short photoperiod, but the process is supervised by JH. Juvenile hormones control development and activity of several other structures and functions of the insect body, such as the fat body. Effects of JHs can be substituted by other naturally occurring sesquiterpenoids in insects called retinoids or by juvenoids. Juvenoids are natural or synthetic analogues of JH. They have different structure and chemical composition (e.g. methoprene) and are commercially used as insecticides.

1.2.3 Peptide neurohormones

Insect neuropeptides are products of neurosecretory cells from central and sometimes peripheral nervous system. Being release into haemolymph they act as regulatory hormones, nevertheless, in nervous system itself they can sometimes work as neurotransmitters or neuromodulators. Many insect neurohormones are pleiotropic, which means they are multifunctional. Their functions are often various. For instance, the same peptide may act both in the CNS and in other tissues, playing different roles in central and peripheral targets (Gäde, 1997). A neuropeptide may modulate the action of classical transmitter and the peptide action may depend on the co-transmitter and the specific circuit where it is released. Neurohormones influence many processes in insect body. They can be divided according their effect to adenotrophic (gland regulating), gonadotrophic, morphogenetic (controlling metamorphosis and morphogenesis), chromotropic (controlling colour change), metabolic (controlling metabolism and homeostasis), myotropic (regulating various muscle contractions), behaviour regulating, etc. A different way of classifying neurohormones is to group structurally related peptides encoded on the same precursor gene.

It the following text just groups of neurohormones, which are somehow involved in the control of reproduction, are mentioned.

1.2.3.1 Gonadotrophic hormones

Gonadotropic peptides are hormones that stimulate or inhibit sexual functions such as ovarian maturation, vitellogenesis, transport of storage substances from the fat body to the ovaries, synthesis of ecdysteroids or gonads development (Gäde, 1997). Nevertheless, a key role is played by juvenile hormones and ecdysteroids. It should be mentioned, that gonadotropic hormones are a bit chaotic group of insect neurohormones with a lot of controversial and contradictory data.

Gonadotropic neurohormones can be divided into two groups, gonadotropins and antigonadotropins. Gonadotropins are stimulating hormones such as ovary maturating parsin (OMP), egg development neurohormone (EDNH) and prothoracicotropic hormone (see below). OMP stimulates secretion of ecdysteroids from ovaries and takes also part in production of vitellogenins. Egg development neurohormone also known as ovarian ecdysteroidogenic factor stimulates development of eggs in ovaries and secretion of ecdysone. Antigonadotropins are inhibiting neurohormones that are responsible for

inhibition of egg development. This group includes hormones like neuroparsin, oostatic hormones and trypsin-modulating oostatic factor. Neuroparsin inhibits the effect of juvenile hormone, stimulates fluid reabsorption of isolated recta, induce an increase lipid and trehalose levels in haemolymph (Wolfner *et al.*, 2005).

1.2.3.2 Metabolic neurohormones

Adipokinetic hormones (AKHs) are peptide hormones that consist of eight to ten residues (except AKH of *Vanessa cardui*, Köllisch *et al.*, 2000), are N-terminally pyroglutamate blocked and display the strongest sequence conservation in the N-terminus (Gäde, 1997; Gäde *et al.*, 2008). AKH peptides are synthesized and released from the cell of the glandular lobe of the corpus cardiacum (CC) (Goldsworthy *et al.*, 1972), an endocrine gland connected to the brain. Since the first identification of AKH-I from the locust *L. migratoria* (Stone *et al.*, 1976), more than 50 AKHs, in several cases also with their specific mRNA have been characterized from a broad spectrum of insect species. AKHs are members of the AKH/RPCH (adipokinetic hormone/red pigment concentrated hormone) family, which comprise peptides both from insects and crustaceans (RPCH). Major AKH function is the control of insect metabolism. They behave as typical stress hormones (Kodrík, 2008) by stimulating catabolic reactions and inhibiting synthetic reactions in order to combat immediate stress problems (e.g. prolong flight). AKHs are responsible for mobilization of lipids from the fat body to haemolymph, increase of haemolymph trehalose levels, stimulation of hearth beat frequency, inhibition of protein synthesis including vitellogenesis (see below in details), inhibition of lipid and RNA synthesis in the fat body, stimulation of trehalose biosynthesis and its mobilization from the fat body and activation of biosynthesis of cytochrome P450 in the fat body (Gäde *et al.*, 1997; Orchard, 1987; Van der Horst, 2003; Van der Horst *et al.*, 2001).

Primary step of the lipid mobilisation during locomotion is a release of AKHs from corpora cardiaca and AKHs binding to a specific AKH G protein-coupled receptor in target cells in the fat body (Gäde & Auerswald, 2002; 2003). The signal transduction mechanism involves stimulation of cAMP production, which depends on Ca^{2+} ions, and following activation of protein kinase cascade. Stored triacylglycerol (TAG) reserves are cleaved by triacylglycerol lipase, resulting in the increased concentration of diacylglycerols (DAG) in the haemolymph (Van der Horst, 2003). Transport of DAG utilizes lipoprotein and high-

density lipophorin, to load the DAG at the fat body cells and to transport the lipid in the haemolymph. At the flight muscles, DAG-derived fatty acids are oxidized for energy generation; the lipophorin is recycled to the fat body thus acting as an efficient lipid shuttle.

In addition to flight, one of the most energy-demanding events in insect life is the production of eggs. Considerable amounts of lipid, protein, glycogen and free carbohydrates have to be provided by the female during vitellogenic growth of the oocytes. Since the inhibition of lipid and protein synthesis in the fat body is controlled by AKHs, AKHs have also importance in regulation of reproductive events. The inhibition of protein synthesis by AKHs is mainly concerning vitellogenin synthesis (Carlisle and Loughton, 1979), the inhibition of other proteins is exceptional (Kodrík, 2008). AKHs heamolymph titre slightly increases when the terminal oocytes are large, thus AKHs inhibit vitellogenesis selectively at the end of the reproductive cycle (Moshitzky and Applebaum, 1990). The inhibition occurs at one tenth of hormone titre of those required for initiation of lipid mobilization in the fat body. The role of AKH in the formation of insect eggs has been experimentally proved by Lorenz (2003) in the cricket *Gryllus bimaculatus*.

Other representatives of the metabolic hormone group are e.g. diuretic hormones that are secreted from corpora cardiaca, thoracic and abdominal ganglia. Diuretic hormones stimulate water reabsorption from Malpighian tubes, primary urine production and diuresis. Antidiuretic hormones are regulating water loss and ions balance, they stimulate water reabsorption from guts to haemolymph. Chloride transport stimulating hormone and ion transport peptide are hormones released from corpora cardiaca that are responsible for balance of ions, especially Cl^- (Nässel, 2002).

1.2.3.3 Morphogenetic neurohormones

The morphogenetic neurohormones primarily control developmental events. However, some of them are more or less indirectly involved also into the insect reproduction. Therefore, some basic data about this neurohormonal group are mentioned in the following paragraph.

Prothoracicotropic hormone (PTTH) is a brain neuropeptide hormone that regulates physiology of the prothoracic gland. PTTH controls moulting processes and metamorphosis by regulating ecdysteroids synthesis. The hormone is usually released by single (pair) cells localized mostly within the pars intercerebralis of the protocerebrum, but the precise site is

often species specific. The same species variability is known for the site of the hormone release into the haemolymph. The release is known as "gated" phenomenon, because the release occurs only during a specific time of the day and only in animals that meet strict criteria (e.g. size, weight).

Allatostatins and allatotropins are neuropeptides from brain neurosecretory cells. Allatotropin stimulates secretion of juvenile hormones. Just few forms of allatotropins have been identified. On the other hand, a lot different allatostatin variants have been identified, nevertheless they all inhibit secretion of the juvenile hormone (Iatrou and Gill, 2004). The background of this discrepancy is not properly understood.

The eclosion hormone (EH) and ecdysis-triggering hormone (ETH) stimulate ecdysis behaviours and physiological changes that occur at the end of the moult. EH is synthesized in ventromedial neurons in the protocerebrum of the brain and is released from corpora cardiaca or ventral nerve cord. EH primary effects CNS. It stimulates eclosion, moulting of larva, pupa and imago through a cascade involving crustacean cardioactive peptide (CCAP), which synchronically stimulates contraction of abdominal muscles. In addition, EH affects plasticity of cuticle, initiates degeneration of intersegment muscles and stimulates production of bursicon. ETH is released from the Inka cells and acts directly on the central nervous system (CNS) to trigger preecdysis and ecdysis behaviour (Žitňan, 1996).

Pheromone biosynthesis activating neuropeptides (PBAN) are a family of neurohormones that stimulate synthesis and production of pheromones in Lepidoptera (Alstein, 2004). The PBAN group is intensively studied due to its potential practical use in control of pest insect populations.

Diapause hormone is produced in suboesophageal ganglion and stimulates embryonic diapause by inhibiting development of the maturing oocytes in the pupal stage (Rafaeli, 2009).

1.3 Experimental model - *Pyrrhocoris apterus* (Heteroptera)

Pyrrhocoris apterus (L.) also known as firebug is a common insect of the family Pyrrhocoridae and the order Heteroptera (true bugs). It is easily recognisable due to its distinct red and black coloration. The distribution of the species is throughout Palaeartic from the Atlantic coast of Europe to northwest of China (Stichel, 1959; Kulik, 1973; Puchkov; 1974). It has been also found in the USA, Central America and India (Barber, 1911).

The firebug has been a convenient experimental insect for biological research for a long time. It has become well known after so called "paper factor" incident in the mid-1960s when *P. apterus* was affected by the presence of juvenoid in a paper commonly used in the laboratory colony (Sláma, 1965 a,b,c).

P. apterus is known to possess two adipokinetic hormones, Pyrap-AKH (1000.4 Da) (Kodrík *et al.*, 2000) and Peram-CAH-II (987.4 Da) (Kodrík, *et al.*, 2002). Both AKH hormones are octapeptides and differ only in one amino acid in position 3. The Peram-CAH-II has a sequence of pGlu-Leu-Thr-Phe-Thr-Pro-Asn-Trp-NH_2 and Pyrap-AKH has a sequence of pGlu-Leu-Asn-Phe-Thr-Pro-Asn-Trp-NH_2.

Vitellogenin in *P. apterus* has been found to consist of two subunits with molecular weight 186 and 150kDa (Socha *et al.*, 1991). Electrophoretic analyses of proteins in *P. apterus* female haemolymph revealed no or just trace Vg level within the first two days after the adult ecdysis. Nevertheless, later on, the Vg level sharply increased reaching maximum on day four; afterwards the Vg level decreased again. Allatectomy of the females resulted in disappearance of the Vg, while application of a juvenoid renewed the production of Vg (Socha *et al.*, 1991) suggesting that Vg production is under the hormonal control in *P. apterus*.

2. Aims

The objective of my Bachelor thesis was to investigate the role of adipokinetic hormone Peram-CAH-II in the insect reproduction and its possible relationship to allatostatins. As a marker of the hormonal action, a level of vitellogenins in *P. apterus* haemolymph female has been monitored and quantified using polyacrylamide gel electrophoresis. As the *P. apterus* allatostatin is not known yet, the commercially available allatostatin III originating from the cockroach *Diploptera punctata* was used for the experiments.

Further clarification of the role of adipokinetic hormones may be potentially useful in order to develop new strategies for controlling pest species.

3. Materials and methods

3.1 Experimental insect

A laboratory stock culture of the firebug *P. apterus* (*L.*) was reared on linden seeds and water at 26 °C under long day photoperiodic regime (18 hours light, 6 hours dark). Freshly emerged females were separated from colonies and subjected to hormonal treatment.

3.2 Hormonal treatment

Freshly emerged adult females (1 day old) were divided into three groups. The first group was injected with 2 µL of a Ringer saline and served as the control group. The second group was injected with 2 µL of allatostatin III solution (Gly-Gly-Ser-Leu-Tyr-Ser-Phe-Gly-Leu-NH_2) (Donley *et al.*, 1993) that contained 5 pmol of the hormone per 1 µL of 20% methanol in Ringer saline. The third group was injected with 2 µL Peram-CAH-II solution (pGlu-Leu-Thr-Phe-Thr-Pro-Asn-Trp-NH_2) (Kodrík *et al.*, 2002) with concentration of 5 pmol per 1 µL of 20% methanol in Ringer saline. In case of the double injection the bugs were injected on day 1 and 3 with 2 µL of Peram-CAH-II solution. The bugs after the hormonal treatment were kept under the same conditions as before ecdysis.

3.3 Sample preparation

Haemolymph of the each treated group was collected daily for four days after the hormone injection, named according to the age of the bugs after the ecdysis (day 2, day 3, day 4 and day 5). In case of preparation of double injected Peram-CAH-II group, the haemolymph for day 3 sample was collected before the second hormonal treatment. Additionally, haemolymph of freshly emerged females without any hormonal treatment was collected (day 1). The haemolymph was collected by cutting off the antennas from at least 5 bugs per sample, catching the leaked haemolymph onto the parafilm and collecting it by pipette in an eppendorf tube cooled on ice. Afterwards, the haemolymph was centrifuged at full speed (18000g) at 4 °C for 10 minutes. The pellet was discarded. Supernatant was transferred into another tube and kept in the freezer. Each haemolymph sample was diluted by sample buffer 1:25. The sample buffer was prepared in situ by 1:1 dilution of the store buffer solution (0.125 M tris(hydroxymethyl)aminomethane pH 6.8, 4% sodium dodecyl sulphate, 20% glycerol in water) with water and adding betamercaptoethanol up to 5 % (e.g. 450 µL store buffer + 50 µL betamercaptoethanol + 450 µL water). Drop of bromophenol

blue was added to each sample as a tracing dye. All samples were boiled for about 3 minutes before the first application on the gel and afterwards were stored in the freezer.

3.4 SDS-PAGE electrophoresis

The SDS-PAGE electrophoresis was performed according to modified procedure of Laemmli (1970) using 1 mm thick 10 % separation gel and 5 % stacking gel in the Mini-PROTEAN Tetra system (BIO-RAD) under denatured conditions. Sample wells were washed with the running buffer used for the electrophoresis. Ten µL of the protein standard (BIO-RAD, M_w = 10, 15, 20, 25, 37, 50, 75, 100, 150 and 250 kDa) and 15 µL of the each sample were loaded in the gel. A typical run took approximately 90 minutes, when during the first 10 minutes 60 V were applied and then the voltage was increased to 120 V. Gels were stained in the staining solution (~ 100 mL) for about an hour and destained in the destaining solution (~ 100 mL) for 2 and half hours (after 1 hour the destaining solution was replaced by the fresh one), while gently shaking. For details, see Tables 1-5.

Table 1: 10 % Separation gel

10 % Separating gel	
30 % Acrylamide	10 mL
1 % Bis-acrylamide	3.9 mL
1M Tris(hydroxymethyl)aminomethane pH 8.7	11.2 mL
Water	4.3 mL
20% Sodium dodecyl sulphate (SDS)	0.15 mL
TEMED	0.01 mL
10 % Ammonium persulphate (APS)	0.1 mL
Total	29.66 mL

Table 2: 5 % Stacking gel

5 % Stacking gel	
30 % Acrylamide	0.99 mL
1 % Bis-acrylamide	1.56 mL
1M Tris(hydroxymethyl)aminomethane pH 6.8	0.75 mL
Water	2.64 mL
20% Sodium dodecyl sulphate (SDS)	0.03 mL
TEMED	0.003 mL
10 % Ammonium persulphate (APS)	0.03 mL
Total	6.003 mL

Table 3: Running buffer

Running buffer	
0.025 M Tris(hydroxymethyl)aminomethane	3 g
0.192 M Glycine	14.4 g
0.2 % Sodium dodecyl sulphate (SDS)	1 g
In water to get 1 L of aqueous solution	

Table 4: Staining solution

Staining solution	
0.07 % Comassie Blue R	0.7 g
7.5 % Acetic acid	75 mL
50 % Methanol	500 mL
In water to get 1 L of aqueous solution	

Table 5: Destaining solution

Destaining solution	
9 % Acetic acid	90 mL
45.5 % Methanol	455 mL
In water to get 1 L of aqueous solution	

3.5 Immunoblotting

Immunoploting procedure was performed according to Towbin *et al.* (1979). Briefly, proteins separated on the gel (see above) with a prestained marker were electrophoretically transferred (100 mA for 30 minutes, 300mA for 1.5 hour) to the nitrocellulose membrane (0.45 μm). The non-specific binding sites were saturated with 5 % skimmed milk solution in phosphate buffered saline with 0.5 % Tween (100 mL PBS + 0.5 mL Tween). The nitrocellulose membrane was then overlaid with the first antibodies against *P. apterus* vitellogenin (Rabbit polyclonal anti-Vg, diluted 1:500 with PBS-Tween – for details see Socha *et al.*; 1991) and incubated for an hour at room temperature. After washing with PBS-Tween (5x5 minutes), the nitrocellulose membrane was incubated with the secondary antibodies labeled with horseradish peroxidase (Goat anti rabbit IgG/Px, diluted 1:1000 with PBS-Tween). Peroxidase was detected by 3,3-diaminobenzidine tetrahydro-chloride in 0.1 M tris(hydroxymethyl)aminomethane pH 7.0 solution in the presence of hydrogen peroxide. For details, see Tables 6 and 7.

Table 6: Immunoblotting buffer

Immunoblotting buffer	
0.025 M Tris(hydroxymethyl)aminomethane pH 8.3	3 g
0.192 M Glycine	14.4 g
0.04 % Sodium dodecyl sulphate (SDS)	0.4 g
20 % methanol	200 mL
0.02 % NaN_3	0.2 g
In water to get 1 L of aqueous solution	

Table 7: Phosphate buffered saline

Phosphate buffered saline (PBS)	
NaCl	45 g
Na_2PO_4 x 12 H_2O	6 g
Na_2HPO_4 x 2 H_2O	1 g
In water to get 0.5 L of aqueous solution	

3.6 Vitellogenin quantification

Gel pictures were taken by the GS-800 Calibrated Densitometer (BIO-RAD). The vitellogenin bands were selected and analysed by using Quantity One program analysis. The area and the optical density of the vitellogenin protein band were measured. Subsequently, the relative optical density of the band was calculated by multiplying the optical density with the band area, which was proportional to the relative amount of vitellogenin.

3.7 Data presentation and statistical analysis

Three different samples of each group (control, allatostatin III, Peram-CAH-II groups) were prepared. Three or four SDS-PAGE electrophoreses followed by the vitellogenin quantification were performed for each sample in order to get 11 or 12 repetitions ($n = 11$ or $n = 12$) for each group (control, allatostatin III, Peram-CAH-II groups). Only one sample of Peram-CAH-II double injection group was prepared and it was analysed by PAGE four times ($n = 4$). For more detail information and raw data, see Appendix.

Graphs and tables were constructed in Microsoft Excel 2010 software. Two-way ANOVA together with Tukey's HSD test were used for the statistical analysis of the data.

4. Results

Position of vitellogenin protein band in *P. apterus* haemolymph samples analysed by electrophoretic methods was identified by immunoblotting method (Figure 4). Molecular weight of the vitellogenin was apparently between 150 and 250 kDa, which fits to the literature values 186 and 150kDa (Socha *et al.*, 1991).

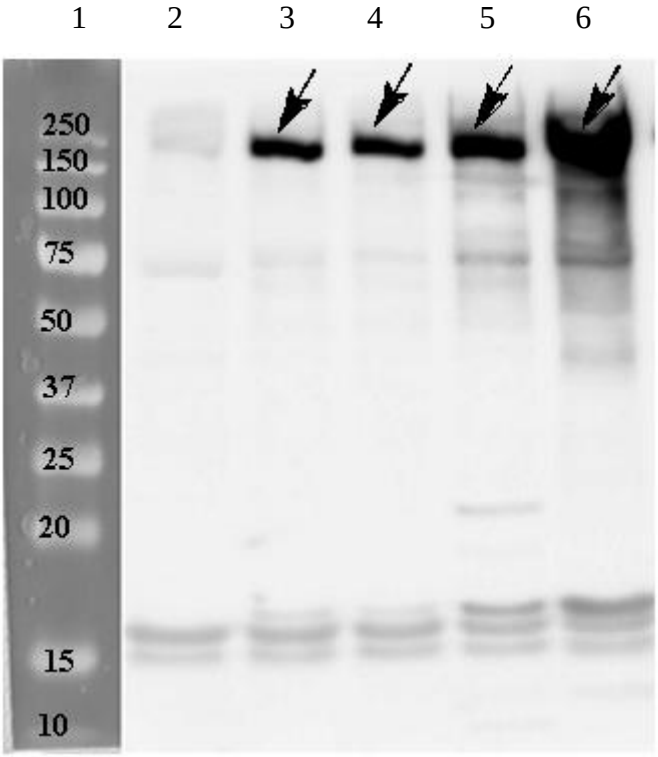

Figure 4: Immunoblotting of haemolymph proteins of *P. apterus* females after the SDS PAGE. Arrows indicate vitellogenins. Legend: 1– protein standards (Bio-Rad, M_W: 10, 15, 20, 25, 37, 50, 75, 100, 150, 250 kDa), 2 – control group day 1, 3 – control group day 2, 4 – control group day 3, 5 – control group day 4, 6 – control group day 5.

The quantification of Vg level in haemolymph of control group of *P. apterus* females (Figure 5 and 9) revealed negligible level of Vg during the first two days of adult development. However, since the day 3 a sharp increase of the Vg level was recorded, reaching the maxima on the last day (5^{th}) of monitoring.

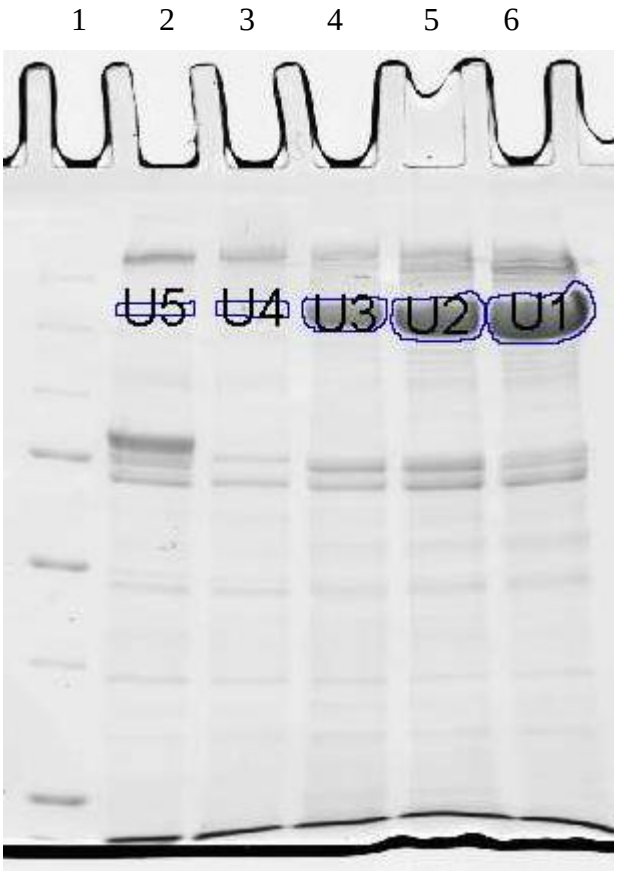

Figure 5: SDS-PAGE gel of control group. Vitellogenins bands are marked U1-U5. Legend: 1– protein standards (Bio-Rad, M_W: 10, 15, 20, 25, 37, 50, 75, 100, 150, 250 kDa), 2 – control group day 1 (U5), 3 – control group day 2 (U4), 4 – control group day 3 (U3), 5 – control group day 4 (U2), 6 – control group day 5 (U1).

Treatment of the *P. apterus* females with allatostatin III showed similar Vg levels during the 2nd and 3rd day of adult development (Figure 6 and 9). Level of Vg during the subsequent days (4 and 5) exponentially increased. The detected Vg level on day 2 was comparable to the control group. On the other hand, Vg levels during following days were lower than those for the control group. Hence, the treatment with allatostatin III resulted in significant decrease in Vg levels of *P. apterus* females from day 3 to day 5 (Figure 9 and Table 8).

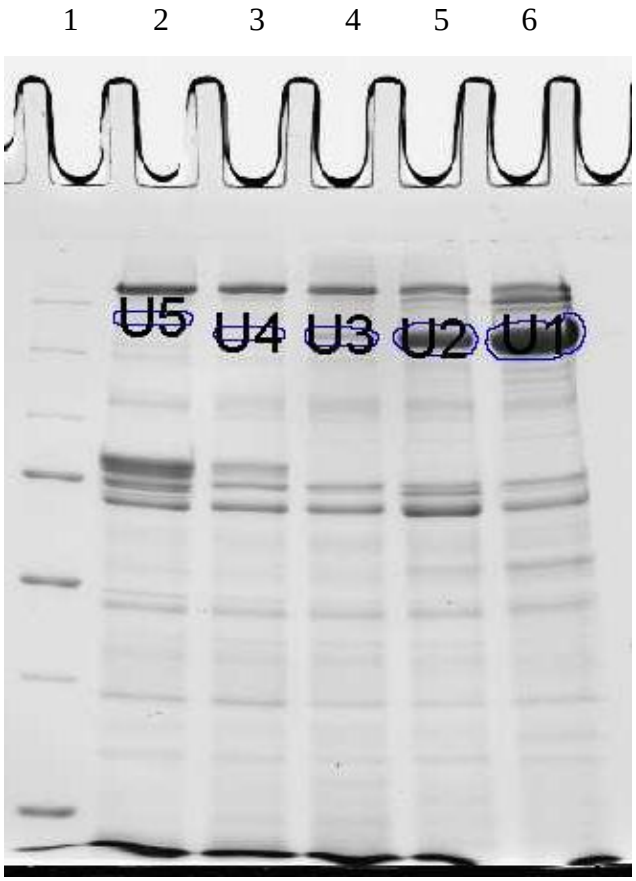

Figure 6: SDS-PAGE gel of allatostatin group. Vitellogenins bands are marked U1-U5. Legends: 1– protein standards (Bio-Rad, M_W: 10, 15, 20, 25, 37, 50, 75, 100, 150, 250 kDa), 2 – control group day 1 (U5), 3 – allatostatin group day 2 (U4), 4 – allatostatin group day 3 (U3), 5 – allatostatin group day 4 (U2), 6 – allatostatin group day 5 (U1).

The Vg quantification of Peram-CAH-II group revealed negligible change of Vg level during the first three days of adulthood (Figure 7 and 9). Recorded Vg levels were raising during the following days (day 4 and 5). Vg levels of the control group were significantly higher than the Vg level after the injection of Peram-CAH-II (Figure 9 and Table 8). Therefore it was assumed, that Peram-CAH-II treatment was responsible for Vg decrease. Vg levels of Peram-CAH-II group were comparable to Vg levels after treatment with allatostatin III. However, on day 4, Vg level in haemolymph in allatostatin group was almost double than that after Peram-CAH-II injection, suggesting that Peram-CAH-II had stronger inhibitory effect.

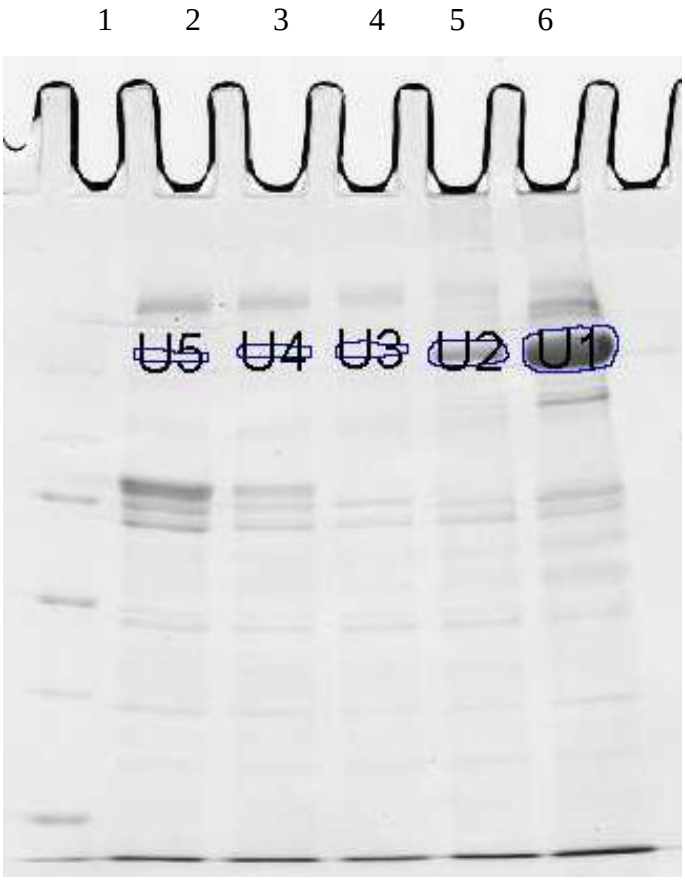

Figure 7: SDS-PAGE gel of Peram-CAH-II group. Vitellogenins bands are marked U1-U5. Legends: 1– protein standards (Bio-Rad, M_W: 10, 15, 20, 25, 37, 50, 75, 100, 150, 250 kDa), 2 – control group day 1 (U5), 3 – Peram-CAH-II group day 2 (U4), 4 – Peram-CAH-II group day 3 (U3), 5 – Peram-CAH-II group day 4 (U2), 6 – Peram-CAH-II group day 5 (U1).

Doubled Peram-CAH-II treatment revealed negligible change of Vg levels from day 2 to day 4 of adult development (Figure 8 and 9). Small increase in Vg level was observed during the 5th day. Peram-CAH-II double injection group had significantly lower Vg level than those recorded for the control group and allatostatin group (Figure 9 and Table 8). Doubled Peram-CAH-II treatment showed no statistical significant difference from single injection of Peram-CAH-II, despite considerable difference visible during the 5th day, where Peram-CAH-II double injection was even more than nine times more effective.

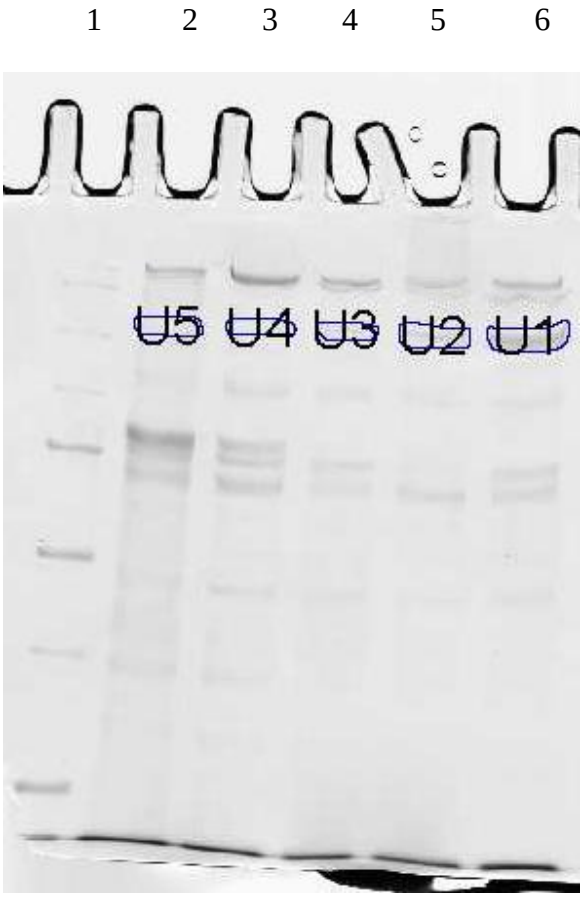

Figure 8: SDS-PAGE gel of Peram-CAH-II group. Vitellogenins bands are marked U1-U5. Legends: 1– protein standards (Bio-Rad, M_W: 10, 15, 20, 25, 37, 50, 75, 100, 150, 250 kDa), 2 – control group day 1 (U5), 3 – Peram-CAH-II double injection group day 2 (U4), 4 – Peram-CAH-II double injection group day 3 (U3), 5 – Peram-CAH-II double injection group day 4 (U2), 6 – Peram-CAH-II double injection group day 5 (U1).

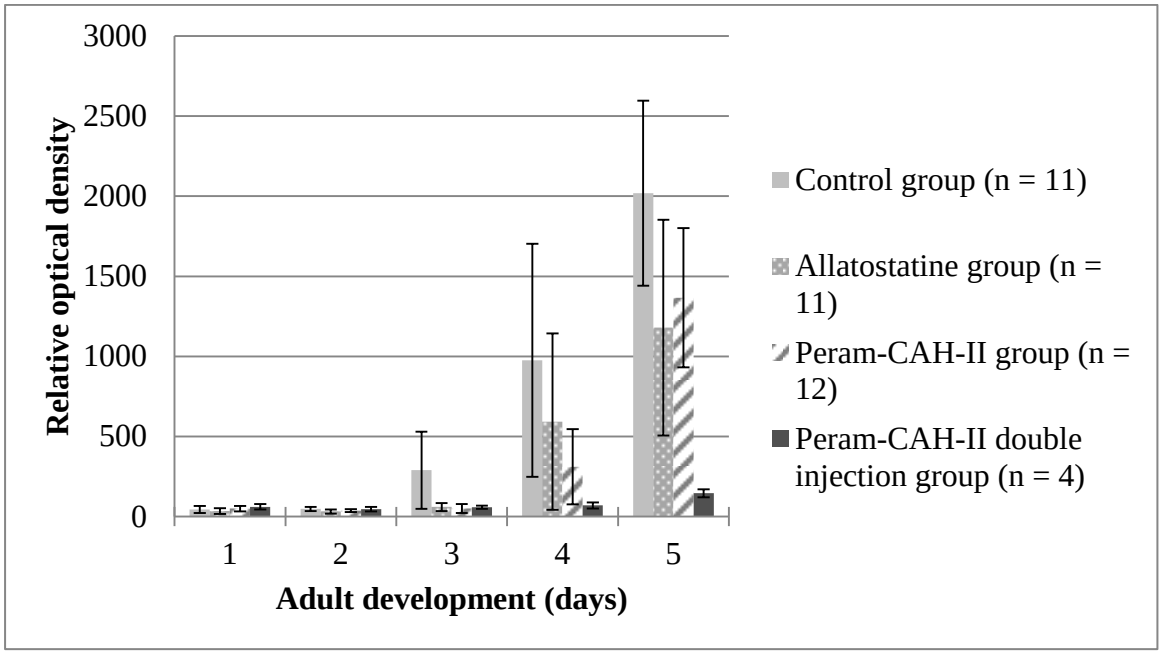

Figure 9: Comparison of relative amounts of vitellogenin proteins in *P. apterus* female haemolymph during the adult development. The hormonal treatment was done on the 1st day; in the case of the double Peram-CAH-II injection on the 1st and 3rd days. For details see Material and methods section. Error bars indicate standard deviations.

Table 8: Two-way ANOVA test together with Tuckey's HSD test of the comparison of the effect of hormonal treatment in different groups

	Mean diff.	95% CI of diff.	Significant
Control group vs. allatostatin group	279.9	30.5 to 529.3	Yes
Control group vs. Peram-CAH-II group	334.2	74.3 to 594.2	Yes
Control group vs. Peram-CAH-II double injection group	583.2	333.8 to 832.7	Yes
Allatostatin group vs. Peram-CAH-II group	54.3	-202.5 to 311.2	No
Allatostatin group vs. Peram-CAH-II double injection group	303.3	57.1 to 549.5	Yes
Peram-CAH-II group vs. Peram-CAH-II double injection group	249.0	-7.9 to 505.8	No

5. Discussion

Insect reproduction is directly depended on the production of the oocytes. The connection between the oocyte growth and the presence of the vitellogenin protein has been described in many papers (Valle, 1993). Vitellogenins are transported from the fat body to the oocytes through haemolymph. Therefore, the levels of the vitellogenin can be detected in haemolymph samples of suitable aged females. The SDS-PAGE electrophoresis with denatured samples was used for Vg analyses. Determination of the Vg levels by measuring the optical density and area of the protein band encounter certain problems. The results showed relatively high deviations, especially during day 4 and day 5. That can be explained by several factors generated by processing and/or biological variability. Some of them definitely occurred during the preparation of samples for quantification, slight differences during staining and destainig procedures, in manual selection of the protein band areas, etc. Additionally, in lower concentration of the vitellogenin (especially day 2 samples), it was difficult to select the band precisely, which was also seen in very weak reaction of the blotting picture in the control group day 2 sample. Nevertheless, also biological factors like differences in age and various speed of development in tested females cannot be excluded.

Molecular mass of vitellogenin of *P. apterus* was reported to be around 180 kDa (Socha *et al.*, 1991) – indeed, both the immunoblotting and also the SDS gels confirmed the results.

In general, the expected changes of Vg levels in female haemolymph were observed. In the control group injected with the Ringer saline only, the Vg levels were exponentially increasing from day 3 of the adult development as the result of intensive oocyte growth. These results correlate with the reported vitellogenin levels of *P. apterus* females (Socha *et al.*, 1991), but with one day delay, most probably due to stress caused by the hormone injections. Therefore, the onset of vitellogenesis appeared on day 3 instead of day 2 after ecdysis and no decrease due to the progressive yolk deposition was observed.

Allatostatins are known to inhibit vitellogenesis secondarily via inhibition of the JH synthesis in corpora allata (Kunkel and Nordin, 1985; Swevers *et al.*, 2005). Accordingly, also synthetic allatostatin III originated from *D. punctata* showed similar effect in *P. apterus* females. Interestingly, its effect was statistically insignificant from that of Peram-CAH-II, despite the inhibitory effect of allatostatin was weaker on day 4. It is documented that allatostatin function is not restricted to the regulation of JH synthesis (Gäde, 1997). It is

supposed allatostatins control also activity of heart (Ude and Agricola, 1995) and gut muscles (Stay *et al.*, 1994) playing important role in "brain-gut" axis.

Double injection of Peram-CAH-II showed significantly lower vitellogenin levels than those in control and allatostatin III groups. The effect of doubled hormonal application was apparently multiplied, nevertheless, the effect did not significantly differ from the single Peram-CAH-II injection probably due to a low differences of Vg levels within the first 3 or 4 days of the observed period.

The Peram-CAH-II results represent the first proof of Vg synthesis inhibition by an adipokinetic peptide representative recorded in *P. apterus* females. Similar effect was recorded previously in various insect species. In *Gryllus bimaculatus*, application of AKH reduced the ovary mass and retarded maturation as a direct consequence of protein and lipid synthesis inhibition in the fat body (Lorenz, 2003). Protein synthesis inhibition in the fat body by AKH has been also demonstrated in *L. migratoria* (Carlisle and Loughton, 1979; Lee and Goldsworthy, 1995a,b). The lipid reserves in the fat body are essential for a normal egg production, as showed in *Aedes aegypti* (Ziegler and Ibrahim, 2001).

6. Conclusion

Reproduction of *P. apterus* can be suppressed by application of the AKH family representative: the inhibitory effect of Peram-CAH-II on vitellogenesis was confirmed. This effect was the most apparent two days after the hormonal treatment (i.e. day 3 of the adult female development). Repeated injection of Peram-CAH-II partially but not significantly intensified the effect. No significant difference between the inhibitory effect of allatostatin III and Peram-CAH-II was detected.

7. References

ALSTEIN, M., 2004: Role of neuropeptides in sex pheromone production in moths (review). *Peptides* **25**, 1491-1501.

BARBER, H. B., 1911: Pyrrhocoris apterus Linn. in the United States. *J. N. Y. Entomol. Soc,* **19**, 111-112.

CARLISLE, J.A., LOUGHTON, B.G., 1979: Adipokinetic hormone inhibits protein synthesis in Locusta. *Nature*, **282**, 420-421.

CHAPMAN, G. B., 1998: Similarity and Reluctance to Trade, *Journal of Behavioral Decision Making*, **11**, 47-58.

DONLEY, B.C., DING, Q., TOBE, S.S., BENDENA, W.G., 1993: Molecular cloning of the gene for allatostatin family of neuropeptides from the cockroach, *Diploptera punctata*, *Proc. Natl. Acad. Csi. USA*, **90**, 8807-8811.

GÄDE, G., 1997: The explosion of structural information on insect neuropeptides. *Fortschr. Chem. Org. Naturst.*, **71**, 1–128.

GÄDE, G., HOFFMANN, K.H., SPRING, J.H., 1997: Hormonal regulation in insects: facts, gaps, and future directions. *Physiol. Rev.*, **77**, 963–1032.

GÄDE, G., AUERSWALD, L., 2002: Beetle's choice – proline for energy output: control by AKHs. *Comp. Biochem. Physiol. B: Biochem. Mol. Biol.*, **132**, 117-129.

GÄDE, G., AUERSWALD, L., 2003: Mode of action of neuropeptides from the adipokinetic hormone family. *Gen. Comp. Endocrinol.*, **132**, 10-20.

GÄDE, G., MARCO, H.G., SIMEK, P., AUDSLEY, N., CLARK, K.D., WEAVER, R.J., 2008: Predicted versus expressed adipokinetic hormones, and other small peptides from the corpus cardiacum-corpus allatum: a case study with beetles and moths. *Peptides*, **29**, 1124–1139.

GOLDSWORTHY, G.J., MORDUE, W., GUTHKELCH, J., 1972: Studies on insect adipokinetic hormones. *Gen. Comp. Endocrinol.*, **18**, 545–551.

GULLAN, P.J., CRANSTON, P.S., 2010: The insect: An Outline of Entomology, 4th edition, *John Wiley & Sons*, 65.

IATROU, K., GILL, S.S., 2004: Comprehensive Molecular Insect Science, **7**, *Elsevier Pergamon*, 337-341.

ISHAAYA, I., NAUEN, R., HOROWITZ, A.R., 2007: Insecticides Design Using Advanced Technologies, *Springer*, 269-279.

KODRÍK, D., SOCHA, R., ŠIMEK, P., ZEMEK, R., GOLDSWORTHY, G.J., 2000: A new member of the AKH/RPCH family that stimulates locomotory activity in the firebug, *Pyrrhocoris apterus* (Heteroptera). *Insect Biochemistry and Molecular biology*, **30**, 489-498.

KODRÍK, D., ŠIMEK, P., LEPŠA, L., SOCHA, R., 2002: Identification of the cockroach neuropeptide Pea-CAH-II as a second adipokinetic hormone in the firebug *Pyrrhocoris apterus*. *Peptides*, **23**, 585-587.

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

KÖLLISCH, G.V., LORENZ, M.W., KELLNER, R., *et al.*, 2000: Structure elucidation and biological activity of an unusual adipokinetic hormone from corpora cardiaca of the butterfly, *Vanessa cardui* . *European Journal of Biochemistry*, **267** , 5502 – 5508 .

KULIK S. A. 1973: The Coreidae and the Pyrrhocoridae (Heteroptera) of the East Siberia and the Far East. In: *Insect Fauna and Ecology of the East Siberia and Far East.*, Irkutsk. 32-43. (in Russian).

KUNKEL, J.G., NORDIN, J.H., 1985: Yolk proteins. In: *Comprehensive Insect Physiology, Biochemistry and Pharmacology*, Eds: KERKUT, G.A., GILBERT, L.J., *Pergamon Pess*, Oxford. 83-111.

LEE, M.J., GOLDSWORTHY, G.J., 1995a: The preparation and use of dispersed cells from fat body of *Locusta migratoria* in a filtration plate assay for adipokinetic peptides. *Analytical Biochemistry*, **228**, 155-161.

LEE, M.J., GOLDSWORTHY, G.J., 1995b: Acetate uptake test; the bases of a rapid method for determining potencies of adipokinetic peptides for structure-activity studies. *J. Insect. Physiol.*, **41**, 191-196.

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

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.

MOSHITZKY, P. & APPLEBAUM, S.W., 1990: The role of adipokinetic hormone in the control of vitellogenins in locusts. *Insect Biochemistry*, **20**, 319 – 323.

NÄSSEL, D. R., 2002: Neuropeptides in the nervous system of *Drosophila* and other insects: multiple roles as neuromodulators and neurohormones. *Progress in Neurobiology*, **68**, 1-84.

NIJHOUT, H.F., 1994: Insect Hormones. *Princeton, University Press*, Princeton, NJ.

ORCHARD, I., 1987: Adipokinetic hormone: an update. *J. Insect. Physiol.*, **33**, 451–463.

PUCHKOV, V.G., 1974: Berytidae, Pyrrhocoridae, Piesmatidae, Aradidae, Tingidae. *The Fauna of Ukraine*, **21**. Akademija Nauk Ukrainskoi RSR. Kiev. 319 pp. (in Ukrainian).

RAFAELI, A., 2009: Pheromone biosynthesis activating neuropeptide (PBAN): Regulatory role and mode of action. *General and Comparative Endocrinology*, **162**, 69- 78.

ROSS, H. H., 1982: A Textbook of Entomology, 4th edition, *John Wiley & Sons*, Chapters 3, 4 and 7.

SAUNDERS, D.S., STEEL, C.G.H., VAFOPOULOU, X., LEWIS, R.D., 2002: Insect Clocks, 3rd edition, *Elsevier Science B. V.*, 163.

SLÁMA, K., 1965a: Effect of hormones on the respiration of body fragments of adult Pyrrhocoris apterus L. (Hemiptera). *Nature*, **205**, 416-417.

SLÁMA K. 1965b: Effect of hormones on growth and respiratory metabolism in the larvae of Pyrrhocoris apterus L. (Hemiptera). *J. Insect Physiol.*, **11**, 113-122.

SLÁMA, K., 1965c: Effect of hormone mimetic substances on the ovarian development and oxygen consumption in allatectomized adult females of Pyrrhocoris apterus L. (Hemiptera). *J. Insect Physiol.*, **11**, 1121-1129.

SOCHA, R., ŠULA, J., KODRÍK, D., GELBIČ, I., 1991: Hormonal control of vitellogenin synthesis in *PYRRHOCORIS APTERUS (L.)* (HETEROPTERA). *J. Insect Physiol.*, **37**, 805-816.

SOCHA, R., ŠULA, J., KODRÍK, D., 2005: Characterisation of accessory gland and haemolymph proteins in adult males of *Pyrrhocoris apterus* (Heteroptera, Insecta) and their endocrine control, In: *Peptides 2004*, Eds: FLEGEL, M., FRIDKIN, M., GILON, CH., SLANINOVA, J., *Kenes International*, Geneva, 445-446.

STAY, B., TOBE, S.S., BENDENA, W.G., 1994: Allatostatins: identifications, primary structures, functions and distribution. *Adv. Insect Physiol.*, **25**, 267-337.

STICHEL, W., 1959: *Illustrierte Bestimmungstabellen der Wanzen. II. Europa.*, **4**, Heft 10. Berlin-Hermsdorf, 289-320.(In German)

STONE, J.V., MORDUE, W., BETLEY, K.E., MORRIS, H.R., 1976: Structure of locust adipokinetic hormone, a neurohormone that regulates lipid utilization during flight. *Nature*, **265**, 207–221.

SWEVERS, L., RAIKHEL, A.S., SAPPINGTON, W., SHIRK, P., IATROU, K., 2005: Vitellohenesis and Post-Vitellogenic Maturation of the Insect Ovarian Follicle, In: Comprehensive Molecular Insect Science, Eds: GILBERT, L.I., IATROU, K., GILL, S.S., *Elsevier Pergamon*, **1**, 87-155.

TOMASCHKO, K.-H., 1999: Nongenomic effects of ecdysteroids. *Arch. Insect Biochem. Physiol.*, **41**, 89-98.

TOWBIN, H., STAEHELIN, T., GORDON, J., 1979: Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose. Procedure and some applications. *Proc. Natl. Acad. Csi. USA*, **76**, 4350-4354.

UDE, J., AGRICOLA, H., 1995: FMRFamide-like and allatostatin-like immunoreactivity in the lateral heart nerve of *Periplaneta americana*: colocalization at the electron-microscopic level. *Cell Tissue Res*., **282**, 69-80.

VALLE, D., 1993: Vitellogenesis in insect and other groups – a review. *Mem. Inst. Oswaldo Cruz*, Rio de Janeiro, **88**, 1-26.

VAN DER HORST, D.J., VAN MARREWIJK, W.J., DIEDEREN, J.H., 2001: Adipokinetic hormones of insect: release, signal transduction, and responses. *Int. Rev. Cytol.*, **211**, 179–240.

VAN DER HORST, D.J., 2003: Insect adipokinetic hormones: release and integration of flight energy metabolism. *Comp. Biochem. Physiol. B: Biochem. Mol. Biol.*, **136**, 217–226.

WIGGLESWORTH, V.B., 1970: Insect Hormones. *W.H. Freeman*, San Francisco.

WOLFNER, M.F., HEIFETZ, Y., APPLEBAUM, S.W., 2005: Gonadal Glands and Their Gene Products, In: Comprehensive Molecular Insect Science, Eds: GILBERT, L.I., IATROU, K., GILL, S.S., *Elsevier Pergamon*, **1**, 179-212.

ZIEGLER, R., IBRAHIM, M.M., 2001: Formation of lipid reserves in fat body and eggs of the yellow fever mosquito, *Aedes aegypti*. *J. Insect Physiol.*, **47**, 623-627.

ŽITŇAN, D., KINGAN, T.G., HERMESMAN, J.L., ADAMS, M.E., 1996: Identification of Ecdysis-Triggering Hormone from an Epitracheal Endocrine System. *Science*, **271**, no. 5245, 88-91.

Appendix

Raw data of relative vitellogenin levels in haemolymph of *P. apterus* females of all experimental groups:

19.2.2013 Control group – 1st set of samples

Sample	Area [mm²]	Density [OD/mm²]	Relative optical density [OD]
Control group day 1	5.11	3.05	15.60
Control group day 2	5.89	3.82	22.48
Control group day 3	7.81	4.37	34.17
Control group day 4	10.10	9.62	97.14
Control group day 5	22.32	79.32	1770.58

13.3.2013 Control group – 1st set of samples

Sample	Area [mm²]	Density [OD/mm²]	Relative optical density [OD]
Control group day 1	3.21	5.07	16.26
Control group day 2	5.16	7.50	38.73
Control group day 3	7.29	7.45	54.28
Control group day 4	10.43	19.28	201.02
Control group day 5	23.08	76.96	1776.63

11.6.2013 Control group – 1st set of samples

Sample	Area [mm²]	Density [OD/mm²]	Relative optical density [OD]
Control group day 1	7.36	7.01	51.61
Control group day 2	5.95	7.87	46.79
Control group day 3	6.87	8.74	60.02
Control group day 4	9.58	13.59	130.21
Control group day 5	18.75	51.30	961.69

13.3.2013 Control group – 2nd set of samples

Sample	Area [mm²]	Density [OD/mm²]	Relative optical density [OD]
Control group day 1	5.58	5.10	28.43
Control group day 2	8.27	4.60	38.09
Control group day 3	12.17	28.40	345.78
Control group day 4	22.32	81.01	1808.11
Control group day 5	26.15	96.32	2518.65

8.6.2013 Control group – 2nd set of samples

Sample	Area [mm²]	Density [OD/mm²]	Relative optical density [OD]
Control group day 1	5.55	9.59	53.26
Control group day 2	8.32	8.21	68.30
Control group day 3	9.44	48.18	455.02
Control group day 4	17.36	112.28	1949.11
Control group day 5	24.39	120.05	2927.60

10.6.2013 Control group – 2nd set of samples

Sample	Area [mm²]	Density [OD/mm²]	Relative optical density [OD]
Control group day 1	7.37	8.82	64.96
Control group day 2	5.47	7.18	39.29
Control group day 3	7.90	20.22	159.74
Control group day 4	15.85	94.45	1497.48
Control group day 5	20.83	112.42	2342.26

11.6.2013 Control group – 2nd set of samples

Sample	Area [mm²]	Density [OD/mm²]	Relative optical density [OD]
Control group day 1	7.75	8.53	66.04
Control group day 2	6.81	9.17	62.43
Control group day 3	10.26	18.34	188.09
Control group day 4	13.67	67.01	916.20
Control group day 5	20.48	86.74	1776.74

21.4.2013 Control group – 3rd set of samples

Sample	Area [mm²]	Density [OD/mm²]	Relative optical density [OD]
Control group day 1	4.93	11.13	54.87
Control group day 2	4.12	11.50	47.45
Control group day 3	11.85	64.91	768.96
Control group day 4	17.41	100.76	1753.90
Control group day 5	23.42	114.25	2675.24

8.5.2013 Control group – 3rd set of samples

Sample	Area [mm²]	Density [OD/mm²]	Relative optical density [OD]
Control group day 1	5.28	4.84	25.52
Control group day 2	6.37	7.12	45.31
Control group day 3	11.10	24.02	266.69
Control group day 4	14.17	38.53	546.06
Control group day 5	24.34	60.16	1464.55

26.5.2013 Control group – 3rd set of samples

Sample	Area [mm²]	Density [OD/mm²]	Relative optical density [OD]
Control group day 1	6.29	6.19	38.93
Control group day 2	6.76	8.11	54.84
Control group day 3	16.11	38.63	622.43
Control group day 4	23.63	59.58	1407.94
Control group day 5	30.67	73.37	2250.60

7.6.2013 Control group – 3rd set of samples

Sample	Area [mm²]	Density [OD/mm²]	Relative optical density [OD]
Control group day 1	10.63	7.75	82.33
Control group day 2	7.45	8.32	61.97
Control group day 3	12.02	18.74	225.36
Control group day 4	16.07	25.88	415.95
Control group day 5	31.54	55.25	1742.49

22.2.2013 Allatostatin group – 1st set of samples

Sample	Area [mm²]	Density [OD/mm²]	Relative optical density [OD]
Control group day 1	4.43	2.77	12.27
Allatostatin group day 2	4.98	2.62	13.05
Allatostatin group day 3	6.52	2.80	18.27
Allatostatin group day 4	8.28	8.56	70.83
Allatostatin group day 5	14.49	20.09	291.10

11.3.2013 Allatostatin group – 1st set of samples

Sample	Area [mm²]	Density [OD/mm²]	Relative optical density [OD]
Control group day 1	4.59	3.72	17.08
Allatostatin group day 2	5.34	2.80	14.96
Allatostatin group day 3	7.71	5.99	46.18
Allatostatin group day 4	17.70	59.26	1048.77
Allatostatin group day 5	20.31	80.59	1636.49

13.3.2013 Allatostatin group – 1st set of samples

Sample	Area [mm²]	Density [OD/mm²]	Relative optical density [OD]
Control group day 1	2.78	4.35	12.10
Allatostatin group day 2	3.40	4.28	14.55
Allatostatin group day 3	5.85	4.84	28.31
Allatostatin group day 4	8.31	19.26	160.06
Allatostatin group day 5	13.42	38.74	520.07

11.6.2013 Allatostatin group – 1st set of samples

Sample	Area [mm²]	Density [OD/mm²]	Relative optical density [OD]
Control group day 1	6.80	8.02	54.55
Allatostatin group day 2	4.92	9.98	49.08
Allatostatin group day 3	6.34	12.09	76.64
Allatostatin group day 4	9.07	10.98	99.58
Allatostatin group day 5	13.25	27.29	361.70

13.3.2013 Allatostatin group – 2nd set of samples

Sample	Area [mm²]	Density [OD/mm²]	Relative optical density [OD]
Control group day 1	5.79	4.85	28.09
Allatostatin group day 2	6.76	4.85	32.79
Allatostatin group day 3	9.93	8.54	84.86
Allatostatin group day 4	19.98	58.99	1178.82
Allatostatin group day 5	22.88	68.90	1576.62

8.6.2013 Allatostatin group – 2nd set of samples

Sample	Area [mm²]	Density [OD/mm²]	Relative optical density [OD]
Control group day 1	5.43	9.35	50.73
Allatostatin group day 2	3.92	7.14	27.99
Allatostatin group day 3	6.75	12.64	85.37
Allatostatin group day 4	16.99	75.68	1285.63
Allatostatin group day 5	21.48	96.71	2076.85

10.6.2013 Allatostatin group – 2nd set of samples

Sample	Area [mm²]	Density [OD/mm²]	Relative optical density [OD]
Control group day 1	8.03	7.39	59.37
Allatostatin group day 2	5.77	6.15	35.47
Allatostatin group day 3	7.93	10.74	85.18
Allatostatin group day 4	19.35	77.76	1505.09
Allatostatin group day 5	22.28	102.87	2292.07

21.4.2013 Allatostatin group – 3rd set of samples

Sample	Area [mm²]	Density [OD/mm²]	Relative optical density [OD]
Control group day 1	4.72	10.57	49.88
Allatostatin group day 2	4.48	11.13	49.88
Allatostatin group day 3	5.32	14.35	76.34
Allatostatin group day 4	9.94	52.31	519.73
Allatostatin group day 5	16.63	84.41	1403.61

8.5.2013 Allatostatin group – 3rd set of samples

Sample	Area [mm²]	Density [OD/mm²]	Relative optical density [OD]
Control group day 1	4.52	5.37	24.28
Allatostatin group day 2	4.85	5.42	26.31
Allatostatin group day 3	5.35	6.05	32.36
Allatostatin group day 4	8.11	10.00	81.11
Allatostatin group day 5	16.34	56.20	918.19

26.5.2013 Allatostatin group – 3rd set of samples

Sample	Area [mm²]	Density [OD/mm²]	Relative optical density [OD]
Control group day 1	5.94	5.39	32.01
Allatostatin group day 2	5.81	6.42	37.31
Allatostatin group day 3	6.79	8.94	60.73
Allatostatin group day 4	13.39	20.88	279.52
Allatostatin group day 5	20.73	45.48	943.05

7.6.2013 Allatostatin group – 3rd set of samples

Sample	Area [mm²]	Density [OD/mm²]	Relative optical density [OD]
Control group day 1	5.31	7.71	40.94
Allatostatin group day 2	5.76	7.58	43.68
Allatostatin group day 3	6.79	8.84	59.97
Allatostatin group day 4	12.27	23.66	290.29
Allatostatin group day 5	19.57	48.74	953.74

19.2.2013 Peram-CAH-II group – 1st set of samples

Sample	Area [mm²]	Density [OD/mm²]	Relative optical density [OD]
Control group day 1	5.43	2.80	15.20
Peram-CAH-II group day 2	7.95	3.02	23.97
Peram-CAH-II group day 3	8.39	3.22	27.05

Peram-CAH-II group day 4	10.22	7.62	77.88
Peram-CAH-II group day 5	18.81	54.07	1016.99

8.6.2013 Peram-CAH-II group – 1st set of samples

Sample	Area [mm²]	Density [OD/mm²]	Relative optical density [OD]
Control group day 1	5.72	8.93	51.07
Peram-CAH-II group day 2	4.83	6.47	31.24
Peram-CAH-II group day 3	4.85	7.23	35.05
Peram-CAH-II group day 4	7.37	14.76	108.78
Peram-CAH-II group day 5	15.56	75.67	1177.44

10.6.2013 Peram-CAH-II group – 1st set of samples

Sample	Area [mm²]	Density [OD/mm²]	Relative optical density [OD]
Control group day 1	6.81	8.29	56.45
Peram-CAH-II group day 2	4.61	4.54	20.92
Peram-CAH-II group day 3	4.43	4.91	21.74
Peram-CAH-II group day 4	8.41	15.59	131.09
Peram-CAH-II group day 5	16.06	69.46	1115.24

11.6.2013 Peram-CAH-II group – 1st set of samples

Sample	Area [mm²]	Density [OD/mm²]	Relative optical density [OD]
Control group day 1	7.89	7.16	56.48
Peram-CAH-II group day 2	6.02	6.49	39.09
Peram-CAH-II group day 3	6.47	6.85	44.31
Peram-CAH-II group day 4	6.82	13.30	90.71
Peram-CAH-II group day 5	17.97	40.44	726.81

16.6.2013 Peram-CAH-II group – 2nd set of samples

Sample	Area [mm²]	Density [OD/mm²]	Relative optical density [OD]
Control group day 1	5.25	7.39	38.82
Peram-CAH-II group day 2	5.03	6.48	32.58
Peram-CAH-II group day 3	6.46	10.53	68.05
Peram-CAH-II group day 4	10.81	41.50	448.82
Peram-CAH-II group day 5	16.66	92.75	1544.92

16.6.2013 Peram-CAH-II group – 2nd set of samples

Sample	Area [mm²]	Density [OD/mm²]	Relative optical density [OD]
Control group day 1	6.72	8.03	53.92
Peram-CAH-II group day 2	7.64	6.61	50.53
Peram-CAH-II group day 3	11.21	10.16	113.91

Peram-CAH-II group day 4	17.79	45.05	801.59
Peram-CAH-II group day 5	25.77	78.59	2025.50

17.6.2013 Peram-CAH-II group – 2nd set of samples

Sample	Area [mm²]	Density [OD/mm²]	Relative optical density [OD]
Control group day 1	8.09	8.63	69.79
Peram-CAH-II group day 2	5.92	7.76	45.98
Peram-CAH-II group day 3	8.44	9.86	83.21
Peram-CAH-II group day 4	16.92	31.32	529.77
Peram-CAH-II group day 5	26.14	56.62	1480.08

17.6.2013 Peram-CAH-II group – 2nd set of samples

Sample	Area [mm²]	Density [OD/mm²]	Relative optical density [OD]
Control group day 1	7.46	8.70	64.93
Peram-CAH-II group day 2	6.26	7.45	46.66
Peram-CAH-II group day 3	6.44	10.55	67.97
Peram-CAH-II group day 4	17.25	31.85	549.47
Peram-CAH-II group day 5	24.90	59.82	1489.34

21.4.2013 Peram-CAH-II group – 3rd set of samples

Sample	Area [mm²]	Density [OD/mm²]	Relative optical density [OD]
Control group day 1	6.01	10.67	64.10
Peram-CAH-II group day 2	3.69	10.26	37.85
Peram-CAH-II group day 3	4.54	9.66	43.83
Peram-CAH-II group day 4	9.78	41.70	407.97
Peram-CAH-II group day 5	19.94	106.80	2130.05

8.5.2013 Peram-CAH-II group – 3rd set of samples

Sample	Area [mm²]	Density [OD/mm²]	Relative optical density [OD]
Control group day 1	5.54	5.22	28.93
Peram-CAH-II group day 2	7.98	5.01	40.02
Peram-CAH-II group day 3	4.94	3.92	19.35
Peram-CAH-II group day 4	7.48	11.31	84.61
Peram-CAH-II group day 5	16.92	63.67	1077.53

26.5.2013 Peram-CAH-II group – 3rd set of samples

Sample	Area [mm²]	Density [OD/mm²]	Relative optical density [OD]
Control group day 1	4.27	7.29	31.14
Peram-CAH-II group day 2	4.90	6.55	32.10
Peram-CAH-II group day 3	5.22	6.54	34.12

Peram-CAH-II group day 4	10.49	23.48	246.28
Peram-CAH-II group day 5	21.05	79.54	1674.43

7.6.2013 Peram-CAH-II group – 3rd set of samples

Sample	Area [mm²]	Density [OD/mm²]	Relative optical density [OD]
Control group day 1	8.73	7.10	61.98
Peram-CAH-II group day 2	6.19	6.62	40.97
Peram-CAH-II group day 3	6.69	7.60	50.89
Peram-CAH-II group day 4	13.19	19.10	251.90
Peram-CAH-II group day 5	21.25	44.08	936.47

16.6.2013 Peram-CAH-II double injection group – 1st set of samples

Sample	Area [mm²]	Density [OD/mm²]	Relative optical density [OD]
Control group day 1	9.10	9.17	83.50
Peram-CAH-II double injection group day 2	6.50	6.74	43.83
Peram-CAH-II double injection group day 3	6.69	10.22	68.38
Peram-CAH-II double injection group day 4	5.46	16.61	90.78
Peram-CAH-II double injection group day 5	8.24	21.80	179.58

16.6.2013 Peram-CAH-II double injection group – 1st set of samples

Sample	Area [mm²]	Density [OD/mm²]	Relative optical density [OD]
Control group day 1	7.06	7.90	55.82
Peram-CAH-II double injection group day 2	6.00	6.53	39.17
Peram-CAH-II double injection group day 3	5.78	7.91	45.69
Peram-CAH-II double injection group day 4	9.04	8.84	79.96
Peram-CAH-II double injection group day 5	10.75	13.49	144.98

17.6.2013 Peram-CAH-II double injection group – 1st set of samples

Sample	Area [mm²]	Density [OD/mm²]	Relative optical density [OD]
Control group day 1	4.94	8.91	43.98
Peram-CAH-II double injection group day 2	4.64	7.78	36.08
Peram-CAH-II double injection group day 3	6.10	8.83	53.84
Peram-CAH-II double injection group day 4	5.46	9.03	49.28
Peram-CAH-II double injection group day 5	9.65	14.25	137.44

17.6.2013 Peram-CAH-II double injection group – 1st set of samples

Sample	Area [mm²]	Density [OD/mm²]	Relative optical density [OD]
Control group day 1	7.22	8.41	60.77
Peram-CAH-II double injection group day 2	9.60	6.87	65.99
Peram-CAH-II double injection group day 3	6.83	9.56	65.25

Peram-CAH-II double injection group day 4	6.68	8.80	58.75
Peram-CAH-II double injection group day 5	9.82	12.32	120.96