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Bachelor Thesis in Biological Chemistry

Role of adipokinetic hormone Pyrap-AKH in insect reproduction

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Annotation:

The effect of Pyrap-AKH on the reproduction of *Pyrrhocoris apterus* was investigated. In the experiments, the attention was focused on the presence or absence, and quantification of vitellogenin – an egg yolk precursor protein – in the haemolymph of females. It was found that Pyrap-AKH similarly as allatostatin III reduces the Vg level in the haemolymph.

Declaration

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Acknowledgement

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List of used abbreviations

AKH – Adipokinetic Hormone
ANOVA – Analysis of Variance
CA – Corpora Allata
CC – Corpora Cardiaca
ELISA – Enzyme-linked Immunoassay
Grybi-AKH – *Gryllus bimaculatus* Adipokinetic Hormone
JH – Juvenile Hormone
Peram-CAH-II – *Periplaneta americana* Cardio-acceleratory Hormone
Pyrap-AKH – *Pyrrhocoris apterus* Adipokinetic Hormone
RPCH – Red Pigment-concentrating Hormone
SDS-PAGE – Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
Vg – Vitellogenin

1 Introduction

Insects such as butterflies and moths have always intrigued humans not just for their beauty and attracting colours but also because of the remarkable changes that occur during their life cycle including reproduction (Gäde et al., 1997). Insect reproduction is controlled by complicated mechanisms involving various hormones. In most insects, vitellogenesis is a process that involves the synthesis of Vg in the female fat body and its transport via the haemolymph into the developing eggs in the ovaries. There the Vg is combined with other substances to form vitellin, a storage protein that is a major component of egg yolk. Vitellogenesis is controlled primarily by juvenile hormone (JH), however, several other hormones including adipokinetic ones (AKHs) are involved as well. In a majority of insect species, JHs are responsible for the synthesis of Vg while AKHs are responsible for its termination. In this thesis, the effect of Pyrap-AKH (Kodrík et al., 2000) on Vg production in the fire-bug *Pyrrhocoris apterus* females was tested, and its activity was compared with the allatostatin III. The female bugs were treated with the hormone(s) and subsequently the presence of Vg in their haemolymph was monitored using the SDS-PAGE.

1.1 *Pyrrhocoris apterus* (Heteroptera)

The fire-bug, *Pyrrhocoris apterus* (L.) (**Fig. 1**) is a popular model species for insect biological research, notably insect metamorphosis and juvenile hormone activity (Socha, 1993). *P. apterus* (Heteroptera: Pyrrhocoridae) which is the experimental animal for this study can be recognized by its red and black colours creating a typical pattern, that serve as a warning colouration. It is adapted to host plants in the order Malvales (Slater, 1982; Ahmad & Schaefer, 1987; Zrzavy, 1990; Schaefer, 1993). *P. apterus* mainly feeds on the seeds of mallow plants (family Malvaceae) mostly trees *Tilia cordata* and *T. platyphylos*. It avoids direct sunlight during the day and is mostly found in the grass, leaf litter and on the ground near host trees and their trunk bases (Zemek & Socha, 2010; Hodgson, 2008). *P. apterus* is a native to Central Europe but its distribution is also predominantly throughout the Palaearctic region from the Atlantic coast of Europe to northwest China. Also, it has been reportedly found in the USA, Central America and India (Socha, 1993). In its immature form, it is usually seen to form aggregates (**Fig. 2**) with tens to hundreds individuals. The representatives of family Pyrrhocoridae are large brightly colored robust insects. The color variation depends on the content of various pigments such as melanin and pterins (Socha,

1993). Melanin which is a basic pigment is responsible for cuticular colouration with its amount being largely increased in melanized forms (Schulze, 1918; Henke, 1924; Seidenstücker, 1953).

One of the main reasons why *P. apterus* is useful as a model species for biological research is its easy laboratory breeding. The interest in it grew in the mid-1960s because of the discovery of the "paper factor" – the substance with juvenile hormone activity (Sláma & Williams, 1966). Earlier interest in it in 1891 by German zoologist Herman Henking led to the first report on sex chromosomes in the history of genetic research. Since then, its morphology, physiology, endocrinology and genetics have been widely studied (Socha, 1993). Other areas of research of the fire-bug are in the testing of newly synthesized insecticides and chemosterilants.

Fig. 1: The fire-bug *Pyrrhocoris apterus*

Fig. 2: Firebugs showing aggregation behavior.

1.1.1 Description

P. apterus is a flightless bug with long-winged and short-winged morphs (Socha, 1993). Its reproduction and wing length pattern are influenced by photoperiod and temperature (Hodek, 1968; Honěk, 1976; Socha, 2001). The most common fire-bug short-winged morph is 6.5-12 mm long, and its females are generally slightly longer and wider with one red band at the posterior end while the males have two red bands. The fore wings usually red with black spots are variable in size, ranging from shortened to absent. The wings are crossed over the back and held flat against the body when at rest. Their antennae are at least half the length of the body. They have prominent eyes at the side of the head (Hodgson, 2008).

1.1.2 Life Cycle

P. apterus typically has one generation (exceptionally two) per year, though some adults can live for more than one year. It undergoes simple metarmorphosis of egg to nymph to adult, depending on the temperature; their entire lifecycle can be up to 2 to 3 months (Hodgson, 2008). In total, the larvae pass through 5 instars. Under long-day conditions (18 hour light, 6 hour day and 26°C), the period taken by the initial four larval instars is 10-14 days, while the final instar last from 7 to 10 days (Socha, 1993). Adults begin mating within a week of emergence. When the day length is reduced to about 12 hours and less per day, adults enter a resting stage called *diapause* (Hodgson, 2008) which can be terminated and re-induced several times throughout a bug's adult life (Socha, 1993).

1.1.3 Eggs and Embryogenesis

Newly laid eggs of *P. apterus* are ovoidal, white or off-white in colour which turns yellow-red near the end of embryonic development in 10 to 14 days before hatching (Socha, 1993; Hodgson, 2008). The mean egg size of *P. apterus* decreases with age and this decrease in egg size affects the quality of the offspring. The egg size determines almost 50% of the variation in body size. The probability of the survival of larvae from a large egg offspring is greater than that of larvae from small eggs due to cannibalistic contests that are usually won by the larger offspring (Honěk, 1992). Several authors (Merlini & Mondelli, 1962; Merlini & Nasini, 1966; Smith & Forrest, 1969) studied the pteridine content in wild-type adult bugs and their eggs. They discovered erythropterin, isoxanthopterin, violapterin and 7-methylxanthopterin in the adults and a 6-substituted derivative of violapterin, in the developing eggs. As embryogenesis progresses, the number and concentration of pteridines increases. As a result of the accumulation of erythropterin and isoxanthopterin, the colour of the egg becomes red at the end of embryonic development (Smith & Forrest, 1969; Socha & Némec, 1992).

1.2 Insect Hormones

A vast number of physiological, developmental, and behavioral events in insects are regulated by hormones. There are two main classes of insect hormones, the *true hormones* –

ecdysteroids or juvenile hormones – which are produced by epithelial glands and the *neuropeptide hormones* produced by neurosecretory cells (Gäde et al., 1997).

The development and reproduction of insects are largely regulated by juvenile hormones and ecdysteroids. These hormones control molting and metamorphorsis of insects in their larval stages while in adult insects, they are involved in the regulation of oogenesis and vitellogenesis in females, and in males are involved in spermatogenesis and growth of the accessory reproductive glands (Koeppe et al., 1985; Nijhout, 1994; Riddiford, 1994; Gäde et al., 1997). Juvenile hormones are synthesized and secreted from the corpora allata while ecdysteroides mostly from prothoracic glands, nevertheless, in certain developmental stages also from other tissues. Certain neurohormones are involved in control of reproduction as well.

1.2.1 Ecdysteroids

Ecdysteroids include a collection of ecdysone and its homologues e.g. 20-hydroxyecdysone. They are molting hormones in mandibulate arthropods (Chang, 1993). They also occur in other invertebrates where they perform functions other than molting (Franke & Käuser, 1989) for example as sex hormones. Ecdysone is a classical steroid hormone with the usual four-ring nucleus of steroids and a full side chain of cholesterol (Gäde et al., 1997). It usually has a keto group conjugated to a double bond as well as five hydroxyl groups (Koolman, 1990).

Ecdysone controls the regulation of several physiological and biological processes related to molting in embryos, larvae, and nymphs and also to the reproduction in imagoes. The true molting hormone has been shown to be 20-hydroxyecdysone (Gäde et al., 1997). A group of ecdysteroids called *phytoecdysteroids* were described in plants where they are acting as toxic substances and/or as feeding deterrents to protect the plants from herbivores (Lafont et al., 1991). The main site of ecdysone synthesis in insect larvae is the prothoracic gland. A variety of stimuli causes the release of an ecdysiotropic neurohormone i.e. prothoracicotropic hormone (PTTH) from the brain which triggers the prothoracic gland to secrete ecdysone by exocytosis into the haemolymph (Gäde et al., 1997).

1.2.2 Juvenile Hormone

Juvenile hormones (JHs) are unique humoral agents, sesquiterprenes (**Fig. 3**) that play important roles in almost every stage of insect development and reproduction (Gäde et al., 1997). Studies in a variety of insects during the past decades have deepened our knowledge and understanding of JH physiology. It has been shown that the JH affects vitellogenesis in the majority of insect species (Engelmann, 1979; Davey, 1983; Postlethwait & Gorgi, 1985). In a number of insects, the synthesis of Vg which is a precursor of vitellin, has been found to occur in the fat body under the control of JH (Engelmann, 1970; Kunkel & Nordin, 1985). JH is synthesized in the corpora allata, a pair (or impair) of endocrine glands behind the brain (Gäde et al., 1997). In *P. apterus*, the synthesis of Vg is also dependent on the presence of JH (Socha et al., 1991). JH affects the production and/or uptake of Vg into the growing oocytes (Gäde et al., 1997). Studies have shown that allatectomy blocks the uptake of Vg while the application of JH restores the uptake of Vg. Allatectomized females laid a low number of eggs; however, the administration of JH analogs such as methoprene restored Vg synthesis and egg production (Gäde et al., 1997). It is known that JH binds to specific receptors in the cytosol and/or nuclei of the target tissue thereby directing the tissue-specific transcription of the Vg genes (Jindra et al., 2013).

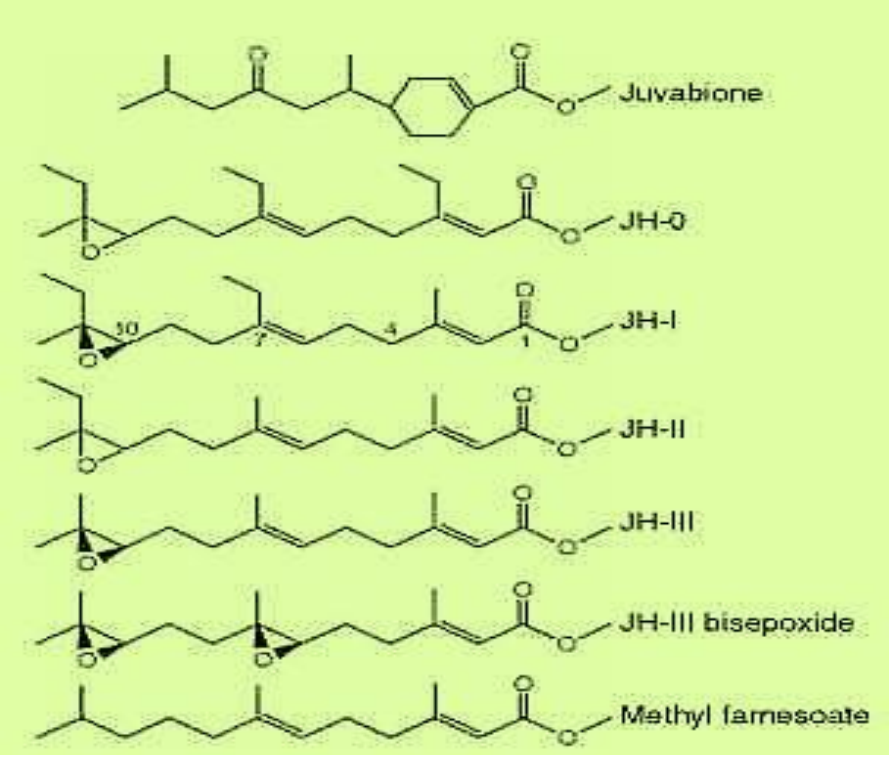


Fig. 3: Structure of natural JHs and their homologs.

Transcription is initiated when the hormone-receptor complex formed is recognized by specific DNA sequences near the Vg genes (Gäde et al., 1997).

JH blocks metamorphosis and control switches between alternative pathways of development at several stages in the life cycle. Generally, the presence of JH during a crucial period implies that no developmental switch occurs and the current developmental state is maintained. The absence of JH during a crucial period leads to changes in gene expression, and the beginning of new developmental processes that launch the insect on a new pathway to development (Gäde et al., 1997).

The juvenile hormone level in haemolymph may change dramatically over short periods of time. The finite haemolymph or tissue levels are a balance between the rates corpora allata biosynthesizes of the hormones and the rate at which the molecules are metabolized or excreted. Although, the physiological levels of JH are known to be determined by JH-degrading enzymes, it is possible that the overall control of tissue and haemolymph levels is more intimately linked with the rate changes of JH biosynthesis (Weaver et al., 1998).

The biosynthesis of JH in corpora allata is regulated by both hormonal and neural inputs (Rachinsky & Tobe, 1996). The hormonal signals may either be stimulatory (allatotropins) or inhibitory (allatostatins) depending upon the species and developmental stage. The signals may reach the glands via the haemolymph (hormones) or through the nervous connections (nervous excitation) (Stay et al., 1994a)

In the haemolymph, JH binds to JH-binding protein(s) which appear to be essential for the maintenance and distribution of JH in the insect body. The regulation of JH is done by a balance between its synthetic rate and degradation. The levels of JH in specific tissues are reduced by degradation ensuring that the JH is maintained at a low level even when its synthesis is not completely stopped (Gäde et al., 1997).

1.2.3 Neuropeptides

Neuropeptides are a large group of hormones involving dozens (hundreds) of peptidic representatives. They control practically all aspects of insect life – metabolism, metamorphosis, development, reproduction, muscle contraction, colour changes and others.

From practical reasons just neurohormones connected with the topic of this thesis are mentioned in the following text.

1.2.3.1 Allatotropin(s)

Allatotropins are neuropeptides that stimulate the production of JH by the CA of insects (Gäde et al., 1997; Audsley et al., 2008). Only a few allatotropins have been identified so far; there are two structurally unrelated allatotropins that have been identified in moths, Manse-AT and Spofr-AT2. Manse-AT increases the synthesis of JH in adults but does not affect the activity of CA in the larvae or pupae of *Manduca sexta* (Hoffmann et al., 1999). Further, it triggers also the contractions of the heart muscles and gut peristalsis in adults while inhibiting the transport of ions across the midgut of the larvae (Audsley et al., 2008).

1.2.3.2 Allatostatin: Their Role in the Regulation of JH Synthesis

Allatostatins are pleiotropic neuropeptide hormones occurring in insects and crustaceans: many types of allatostatins have been isolated so far. Their main function is the inhibition of the synthesis of JH. They all act rapidly and reversibly and occur in all groups of insects studied. However, they act as inhibitors of JH production in only some groups of insects (Stay & Tobe 2007). Allatostatins were localized immunocytochemically and occur in lateral neurosecretory cells of the brain which are neurally connected with the corpora allata. Their release from the neurons is supposed to be paracrine. Allatostatins has been found to also occur in the haemolymph of cockroaches acting on the corpora allata through this pathway. Experiments have suggested that the major factors regulating JH synthesis in the cockroaches are as a result of the changes in the release of allatostatins and in the sensitivity of corpora allata to them (Stay et al. 1996).

Allatostatin's ability to inhibit corpora allata depends on the concentration of the peptides and also on the sensitivity of this gland to them (Lorenz, 2003). Four different allatostatin inhibiting JH synthesis have been isolated from brains of the virgin female cockroach *Diploptera punctata*. These allatostatins which are 8-13 amino acids long are amidated showing sequence similarity such as a 3-amino acid sequence at the C-terminal end common to all four peptides. They are – **Allatostatin I**, Ala-Pro-Ser-Gly-Ala-Gln-Arg-Leu-Tyr-Gly-Phe-Gly-Leu-NH_2; **Allatostatin II**, Gly-Asp-Gly-Arg-Leu-Tyr-Ala-Phe-Gly-Leu-NH_2;

Allatostatin III, Gly-Gly-Ser-Leu-Tyr-Ser-Phe-Gly-Leu-NH_2; and **Allatostatin IV**, Asp-Arg-Leu-Tyr-Ser-Phe-Gly-Leu-NH_2 (Woodhead et al. 1989). Allatostatin III was used in the experiments of this thesis.

An ELISA and immunocytochemistry techniques have revealed that allatostatins are not only found in the insect brain but also in many peripheral tissues such as e.g. the gut (Reichwald et al., 1994). Allatostatins are therefore an example of "brain-gut peptides" and their role may not be confined to the control of the biosynthesis of JH. The action of allatostatin on the antennal heart muscle is likely done to balance the action of another peptide, most likely proctolin (Gäde et al., 1997). At certain phases of the vitellogenic cycle, a strong neutrally mediated inhibition of the CA has been recorded. It has been shown that the release of allatostatin can be inhibited by the growing ovaries leading to an increase in JH synthesis in vitellogenic females (Gäde et al., 1997).

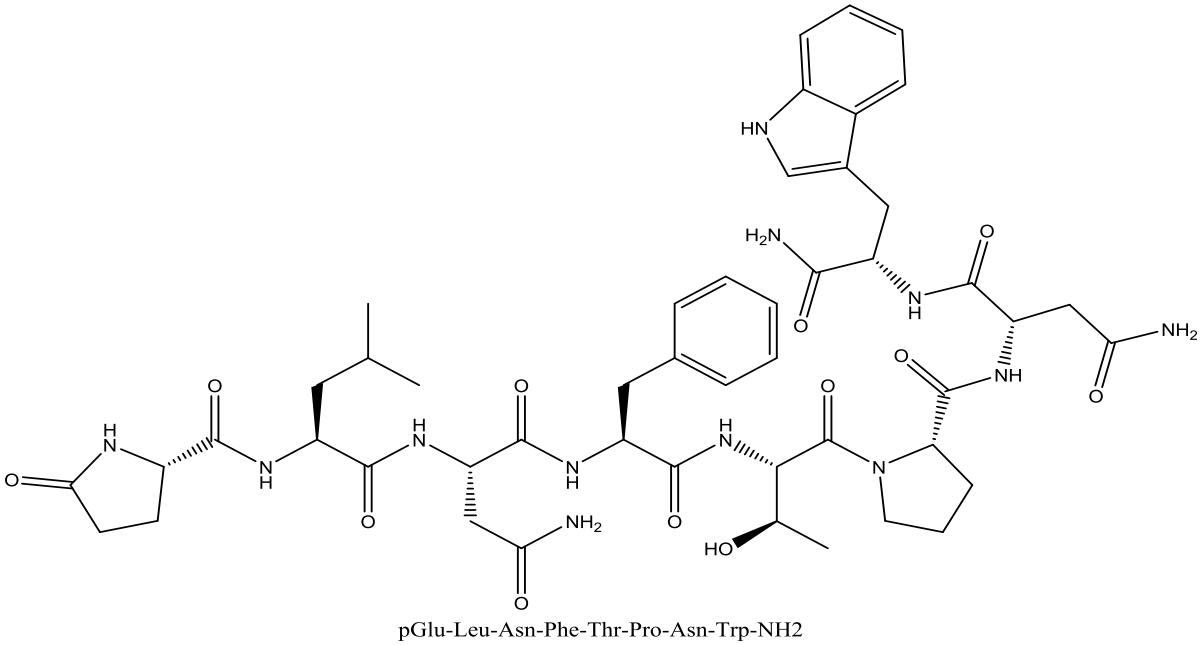
1.2.3.3 Adipokinetic Hormones and Their Metabolism

Adipokinetic hormones (AKHs) are important group of insect metabolic neuropetides. They are synthesised, stored and released by neurosecretory cells from the corpora cardiaca (CC), a neuroendocrine gland connected to the brain (Fernlund & Josefsson, 1972). More than 50 insect AKHs have been identified so far (Kodrík, 2008). AKHs are octa-, nona- or decapeptides with the N-terminus blocked by a pyroglutamate residue, while the C-terminus is blocked by an amide (Gäde et al., 1997). Most importantly, AKHs control the energy metabolism and mobilization of different kinds of energy reserves such as lipids, carbohydrates and/or certain amino acids (Gäde et al., 1997). They are pleiotropic in that there are other actions connected to their metabolic role (Kodrík, 2008). They generally act as typical stress hormones, stimulating catabolic reactions and inhibiting synthetic reactions to combat stress and to suppress processes that are momentarily less important (Kodrík, 2008). AKHs are released from the CC during energy demanding events such as flight to induce the mobilisation of energy stores in the fat body, thereby increasing the levels of diacylglycerol, trehalose and / or proline in the haemolymph (Goldsworthy & Mordue, 1989; Candy et al., 1997; van der Horst et al., 2001). When the peptides are released into the haemolymph, they can then be transported to their target cells in order to bind to specific membrane-bound receptors and apply their biological functions (Gäde et al., 1997). AKH are peptidic hormones and are therefore not able to freely penetrate the cell membrane, their message is

transferred via specific membrane receptors (Park et al., 2002; Staubli et al., 2002; Hansen et al., 2006; Kaufmann & Brown, 2006). The receptor is attached to a G-protein that triggers two or three biochemical pathways thereby producing energy rich substrates such as proline, trehalose or diacylglycerols which are then transported to the target effectors usually the working muscle cells (Gäde & Auerswald, 2002; 2003).

AKHs also stimulate muscle activity and walking in insects by keeping metabolic processes at levels enough to cover their energy needs (Kodrík et al., 2000). AKH also plays biochemical actions in insect body. Such biochemical roles include the activation of adenylate cyclase, lipase, phospholipase C, glycogen phosphorylase (Spencer & Candy, 1976; Vroenen et al., 1997; Van Marrewijk et al., 1980); triggering of mitochondrial cytoheme biosynthesis (Keeley et al., 1991); inhibition of RNA (Kodrík & Goldsworthy, 1995), protein (Carlisle & Loughton, 1979) and lipid (Gokuldas et al., 1988) syntheses. Some physiological actions of AKHs include the stimulation of heart beat (Scarborough et al., 1984), stimulation of general movement (Socha et al., 1999), improvement of immune responses (Goldsworthy et al., 2002, 2003a), suppression of egg maturation (Lorenz, 2003) and control of starvation-induced foraging behaviour.

In *P. apterus* two adipokinetic octapeptides have been identified: the Pyrap-AKH (pGlu-Leu-Asn-Phe-Thr-Pro-Asn-Trp-NH_2; **Fig. 4**) (Kodrík et al., 2000) used in this study, and the Peram-CAH-II (pGlu-Leu-Thr-Phe-Thr-Pro-Asn-Trp-NH_2) (Kodrík et al., 2002) which differs from the Pyrap-AKH just in one amino acid in position 3 (Asn vs. Thr).



pGlu-Leu-Asn-Phe-Thr-Pro-Asn-Trp-NH2

Fig. 4: Structure of Pyrap-AKH as illustrated by ChemBioDraw Ultra software.

1.3 Insect Reproduction

After fertilization, the egg will produce a new organism. In insects and other oviparous organisms, embryonic growth takes place outside the maternal body. As a result, the survival of the egg and the growth of the embryo depend on the use of stored yolk which is composed of lipids, proteins, sugars and other minor compounds. During development, the yolk will be slowly used depending on the needs of the specific cells introduced by the genetic program of the embryo (Atella et al., 2005).

1.3.1 Vitellins, Vitellogenins and Vitellogenesis

In oviparous organisms, the eggs contain large amounts of yolk, which are utilized by the growing embryo. In insects, the vitellogenesis is a process of vitellin accumulation, and involves massive synthesis of the Vg proteins and their deposition in the developing oocyte in a form of vitellin (Valle, 1993). It is a period of rapid oocyte growth, when the vitellin deposition oocurs (Pan et al., 1969; Hagedorn & Kunkel, 1979; Postlethwait & Giorgi, 1985). The synthesis of Vg occurs briefly and usually involves extensive cytological remodeling of structures responsible both for its synthesis in the fat body and for its uptake in the ovary (Valle, 1993). The expression of Vg and its secretion into the haemolymph by fat body and its uptake by the ovary are regulated by the hormonal and sexual levels (Atella et al., 2005).

1.3.2 Structure of Vitellogenin

Vitellogenins are phospho-lipo-glyco-proteins composed of different subunits that can be found as monomers or dimers with a native molecular weight in the range of 300-600 kD. An exception is a native molecular weight of 200 kD in higher Diptera. The phosphate groups present in Vg are generally either bound to serine or via phospholipids (Valle, 1993). It has also been found that phosphate groups are also associated with mannose as seen in the Vg of *Rhodnius prolixus* (Masuda & Oliveira, 1985). A small portion of the phosphate is also associated with the carbohydrates in the Vg of the moth *Manducta sexta* (Osir et al., 1986b). In insects, the carbohydrates associated with Vg are of the high-mannose type (Ng & Dain, 1976).

The lipids associated with Vg are mainly di- and triglycerides, phosphatidylcholine, phosphatidylethanolamine and cholesterol (Hagedorn & Judson, 1972; Chen et al., 1976; Izumi et al., 1980; Baert et al., 1984)

As mentioned above, Vgs contain several subunits. In most insects, the subunits - range from 50-60 kDa to 150-200 kDa - are derived from precursors with molecular weight around 200 kD. Cleavage of these precursors can also occur at alternative sites to generate subunits with both homologous and specific regions (Chen et al., 1978).

As a result of the heterosynthetic nature of vitellogenesis in insects, their oocytes are exclusively responsible for the accumulation of yolk proteins. Hence, insect oocytes contribute a set of structures aimed to choose, internalize and reserve specific proteins like microvilli, yolk granules, coated pits and coated vesicles (Atella et al., 2005). Under normal circumstances, Vg is present only in the haemolymph of reproductive adult female insects (Socha et al., 1991).

1.3.3 Synthesis and Uptake of Vitellogenin

In the majority of insects, the site of Vg synthesis is the fat body an equivalent of vertebrate liver and adipose tissue. During vitellogenesis, the cells of the fat body undergo significant changes by which they are converted from lipid and glycogen storage cells to cells with a massive protein synthesis (Valle, 1993). From the fat body Vg is transported into the developing eggs via haemolymph. The intake of vitellogenin molecule into the oocyte is provided by selective and receptor-mediated endocytosis (Valle, 1993).

In the oocyte, Vg dissociates rapidly from the receptor and is transported to the endosomes. The fusion of these endosomes yields organelles called *yolk bodies* which are specialized in long-term storage and where the protein may be crystallized (Raikhel, 1984). Proteins which are non-specifically internalized are accumulated in vesicles and moved to the lysosomal system (Storella & Kunkel, 1979; Raikhel & Lea, 1986).

1.3.4 Hormonal Control of Vitellogenesis

Hormonal control of vitellogenesis is a complicated event controlled by JH, ecdysteroids and certain nerohormones. In most insect species the principal role is played by JH, however, in dipterans the role of ecdysteroids is enhanced.

Brain signals regulate the synthesis of JH by the CA depending on the nutritional or mating status of the insect. In locusts for example, the production and release of JH can be initiated by mating and some food odors acting via the neurosecretory cells in the brain (Chapman, 1998). Thereafter the JH affects directly the Vg production in fat body cells. This JH activity is a typical example of hormonally controlled gene expression.

In Dipterans, the JH also participates in the Vg production; however, ecdysteroids are responsible for the imminent Vg gene expression (Chapman, 1998). In mosquito, *Aedes* females e.g., the synthesis of ecdysone is controlled by the egg development neurosecretory hormone which is triggered by a blood meal and produced by brain cells. This hormone stimulates the follicle cells to produce ecdysone if JH has rendered it capable to respond. Ecdysone is converted then to an active hormone 20-hydroxyecdysone in the fat body (Chapman, 1998).

As already mentioned above AKHs also play an important role in egg production (Lorenz, 2003). They selectively inhibit vitellogenesis at the end of the reproductive cycle when the haemolymph titre of AKHs increases when the terminal oocytes are large (Moshitzky & Applebaum, 1990). The inhibition of Vg does not affect lipid mobilization because it occurs at hormone levels that are about one tenth of those needed to trigger lipid mobilization in the fat body (Carlisle & Loughton, 1986). It has been shown in crickets that injection of Grybi-AKH elicited reduction of protein and lipid content in the fat body and also in the ovary mass because of the slow maturation of the oocytes and lesser number of the terminal oocytes (Lorenz, 2003).

2 Goals

The main goal of the thesis was to study relationships between Pyrap-AKH and allatostatins – in the control of reproduction in the model species of the fire-bug *Pyrrhocoris apterus*. The effect of both hormones was studied through monitoring of vitellogenin level in female

haemolymph after the hormonal treatment. The SDS-PAGE and immunoblotting techniques were employed to identify the vitellogenin bands which were then quantified using a specialized scanner.

3 Materials and Methods

3.1 Experimental Animals

The fire-bugs *P. apterus* (Heteroptera, Insecta) collected from a wild population at Ceske Budejovice (Czech Republic, 49°N) were reared under a long day (18 h light, 6 h dark) photoperiodic regime and fed on linden seeds and water *ad libitum* at 26 ± 1°C (Kodrík et al., 2010). Freshly ecdysed female adults designated for experiments were collected from the stock colony and kept in separate groups in glass jars under the same rearing conditions as the stock colony (Socha et al., 1991).

3.2 Insect Handling and Hormonal Treatments

The freshly emerged female adults designated as day 1 were used for the hormonal injections. The injections were made ventrolaterally through the intersegmental membrane between the abdominal segments in the direction of the head (Kodrík et al, 2000). Each female received 10 pmol Pyrap-AKH (pGlu-Leu-Asn-Phe-Thr-Pro-Asn-Trp-NH_2; Vidia Praha) or 10 pmol allatostatin III (Gly-Gly-Ser-Leu-Tyr-Ser-Phe-Gly-Leu-NH_2; Bachem Switzerland) always in 2 µl of 20% methanol in Ringer saline. The control animals were injected with 2 µl of Ringer saline only.

3.3 Collection of Haemolymph

Haemolymph was collected from all experimental groups at day 1 (before the injection) and then at days 2, 3, 4 and 5 after the adult ecdyses. To collect haemolymph, the antennas of the experimental bugs were cut off and the leaking haemolymph was collected on a parafilm (approximately 2 – 3 µl per female). The haemolymph from at least five bugs was centrifuged at 4°C, the supernatant transferred into another Eppendorf tube and stored in a freezer at -20°C until needed.

3.4 Chemicals Used and Preparation

Table 1: Hormones used for the insect treatment, and reagents used for the SDS-PAGE

Hormone Preparation	
Pyrap-AKH in 20 % methanol in Ringer saline - concentration 5 pmol/µl	For 1000 µl, 28.73 µl of stock (174.05 pmol/µl) + 921.27 µl 20% methanol in Ringer.
Allatostatin III in 20 % methanol in Ringer saline - concentration 5 pmol/µl	For 1000 µl, 5 µl of stock (1 nmol/µl) + 995 µl Ringer saline solution
20 % methanol in Ringer saline	For 10 ml, 2 ml methanol + 8 ml Ringer saline
Sample Preparation	
Sample buffer (store solution)	0.125 M tris pH 6.8, 4% SDS, 20% glycerol in water
Sample buffer	Prepared from store buffer by diluting 1:1; β-mercaptoethanol is added up to 5% (e.g. 450µl store solution + 50µl beta-mercaptoethanol + 500µl water)
Gel Preparation	
10% separating gel	30% acrylamide 2 ml, 1% bis-acrylamide 0.78 ml, 1M tris pH 8.7 2.24 ml, water 0.86 ml, 20% SDS 30 µl, 10% amonium persulfate 20 µl, TEMED 2 µl.
5% staking (focusing) gel	30% acrylamide 0.33 ml, 1% bis-acrylamide 0.52 ml, 1M tris pH 6.8 0.25 ml, water 0.88 ml, 20% SDS 10 µl, 10% amonium persulfate 10 µl, TEMED 1 µl. The gel should polymerizes at least 1 hour before using.
Acrylamide (BioRad) 30 %	30 g acrylamide, 100 ml water
Bis acrylamide 1%	1 g N,N dimethyl bis acrylamide, 100 ml water
1 M tris pH 6.8	24.2 g of Tris, 200 ml water, pH is adjusted

	with 1 N HCl
1 M tris pH 8.8	24.2 g of Tris, 200 ml water, pH adjusted with 1 N HCl
10% ammonium persulfate (freshly prepared)	For 200 µl; 200 µl water and 0.02 g solid APS
10% SDS	For 200 µl; 200 µl water and 0.02 g solid SDS
Running buffer (10x)	0.025M tris (30g), 0.192M glycine (144g), 0.2% SDS (10g) in water (1l).
Staining solution	Coomasie Blue R 0.07% (0.7g), acetic acid 7.5% (75ml), methanol 50% (500ml) in water (up to 1l).
Destaining solution	Acetic acid 9% (90ml), methanol 45.5% (455ml), water (455ml).
Immunoblotting	
Phosphate buffered saline (10x)	For 1 L buffer, 10 mM sodium phosphate, 150 mM NaCl, pH 7.4
Washing solution	PBS + Tween (0.5% Tween 20)
5% skimmed milk	25 g skimmed milk in 500 ml PBS
Primary antibody	Rabbit polyclonal anti-Vg (dilution 1:500)
Secondary antibody	Goat anti rabbit IgG/Px (Sigma) (dilution 1:1000)

3.5 Polyacrylamide Gel Electrophoresis

Modified sodium dodecyl sulphate - polyacrylamide gel electrophoresis (SDS-PAGE) according to Laemmli (1970) was used for the analysis of the haemolymph samples. The analyses were done on the Bio-Rad Mini-Protean Tetra Cell. All reagents used are summarized in **Table 1** above.

3.5.1 Sample Preparation

The thawed haemolymph samples were diluted in a sample buffer 1:25, mixed with a drop of bromophenol blue for tracing and boiled in a heating block for about 3 minutes. Aliquots of 15 µl of the samples were taken for the SDS-PAGE analysis.

3.5.2 Gel Preparation

Five percent stacking and 10% separation gels were used in the SDS-PAGE. For determination of molecular weights of studied proteins the MW markers (Protein M S35, Thermo Scientific) were used. Ten µl of protein marker were taken for the SDS-PAGE. The set of markers contained the following standards: 10, 15, 20, 25, 37, 50, 75, 100, 150 and 250 kDa.

3.5.3 Running Conditions

For the first 15 minutes, the set up was run at 60 V and for the next hour till the end of the electrophoresis, it was run at 120 V

3.5.4 Staining and Destaining

After electrophoresis, the gels were stained in staining solution for about 1 hour, and destained for about 1.5 hours or more.

3.5.5 Immunological Detection of Vitellogenin

Immunoblotting was carried out according to Towbin et al. (1979) as modified by Socha et al. (1991) to confirm the identity of the 150 kDa polypeptide as vitellogenin. After electrophoresis with a pre-stained marker, the separated proteins were electroblotted onto nitrocellulosemembrane. The non-specific binding sites were then saturated with a 5% skimmed milk in phosphate-buffered saline that is containing 0.5% Tween 20 (PBS-Tween). Then the nitrocellulose sheets were overlaid with the first antibody – rabbit polyclonal – against Vg (for the Ab characterization see Socha et al., 1991) with a dilution 1:500 of PBS-Tween. It was then incubated for 1 hour at room temperature. To visualize the antigen-antibody complex, it was incubated for another 1 hour at room temperature in the second

antibody Goat anti rabbit IgG/Px (Sigma) which is a goat antirabbit immunoglobulin G labelled with horseradish peroxidase diluted in PBS-Tween, 1:1000. In 0.1 M tris-HCl at pH 7.0, the substrate for peroxidase, 3,3-diaminobenzidine tetrahydrochloride was dissolved while the reaction was continued for 1-2 min in the presence of hydrogen peroxide.

3.5.6 Quantification of Vitellogenin Band

The destained gels were scanned and the vitellogenin bands were quantified using the GS-800 scanner (Bio-Rad). The quantity was expressed as relative optical density (= area [mm^2] x optical density [OD/mm^2]) of the particular bands.

3.5.7 Statistical Analysis

The analyses of vitellogenin levels in haemolymph were done in three replicates for all hormonal treatments. Thus three independent gels were run for control, Pyrap-AKH and allatostain treated females, respectively. Two-way ANOVA with the Tukey's multiple comparison test was used to evaluate statistical significance of the results (see **Fig. 5)**.

4 Results

Quantification of the Vg level in control *P. apterus* female haemolymph revealed negligible, practically non-detectable level within the first two days of the adult development (**Fig. 5** & **6**). After that at day three a sharp increase of the Vg level was recorded reaching maxima at the end of the observed period (day five). However, the allatostatin treatment (10 pmol) substantially changed the course of the Vg levels (**Fig. 5** & **7**) – the intensive increase was moved to day four and the total increase at day five was significantly lower than that in controls. Nevertheless, the most dramatic changes were recorded after the Pyrap-AKH (10 pmol) treatment (**Fig. 5** & **8**). This treatment radically inhibited the Vg level during almost whole observed period – certain increase was recorded at the day five only. Statistical evaluation using two-way ANOVA with the Tukey's multiple comparison test revealed significant changes after both allatostatin III and Pyrap-AKH treatments on 1% level (**Fig. 5**;

for more information on the crude data used for the statistics - see **Table 2,3** & **4** at the supplement section).

The identity of studied Vg bands was further confirmed by immunoreaction with the anti-Vg antibody as is shown in **Fig. 9**.

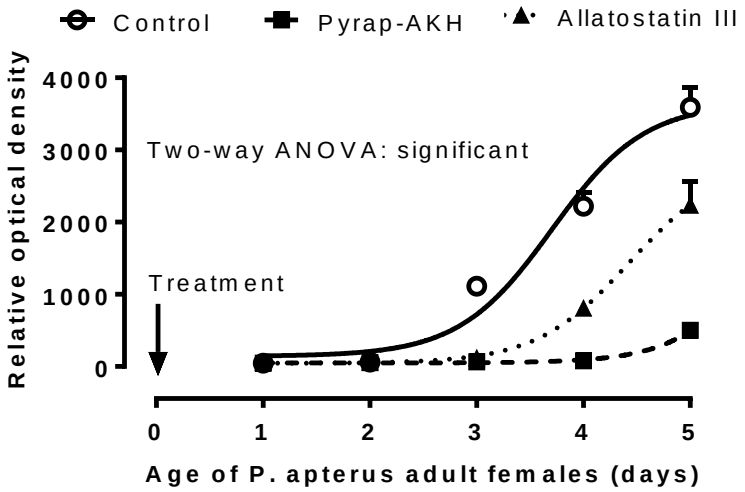


Fig. 5: Comparison of relative level of vitellogenin in female haemolymph in various times after the hormonal treatment. Differences among all experimental groups were statistically significant on 1% level using two-way ANOVA with the Tukey's multiple comparison test.

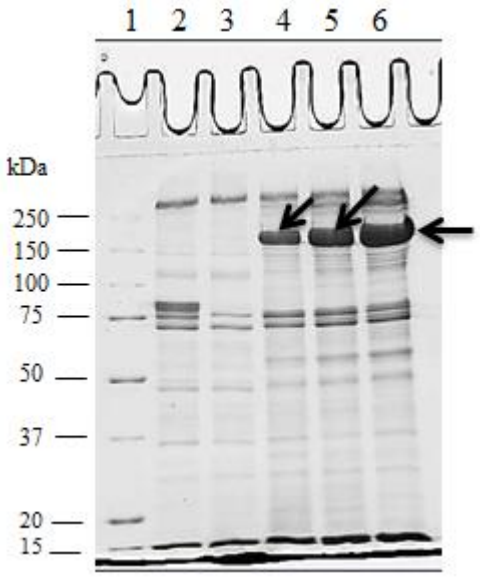


Fig. 6: Example of the SDS-PAGE analysis of *P. apterus* female haemolymph monitoring the level of Vg on different days after the Ringer saline (control) treatment. Arrows indicate Vg. Lane 1 – MW markers; Lane 2 – day 1 without any treatment; Lane 3 – day 2 after treatment; Lane 4 – day 3 after treatment; Lane 5 – day 4 after treatment; Lane 6 – day 5 after treatment.

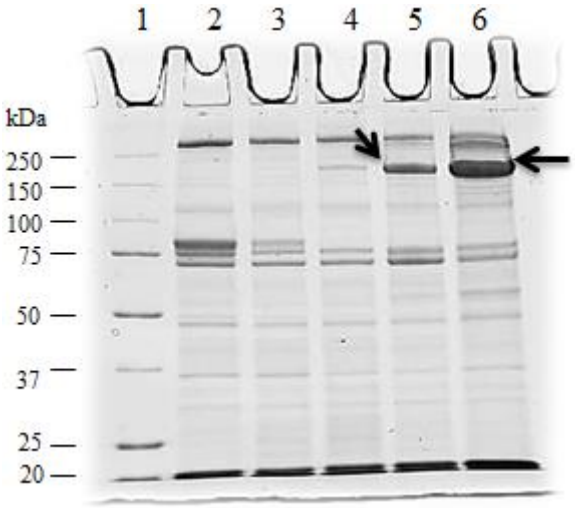


Fig. 7: The SDS-PAGE analysis of *P. apterus* female haemolymph monitoring the level of Vg on different days after the allatostatin treatment. Arrows indicate Vg. Lane 1 – MW markers; Lane 2 – day 1 without any treatment; Lane 3 – day 2 after treatment; Lane 4 – day 3 after treatment; Lane 5 – day 4 after treatment; Lane 6 – day 5 after treatment.

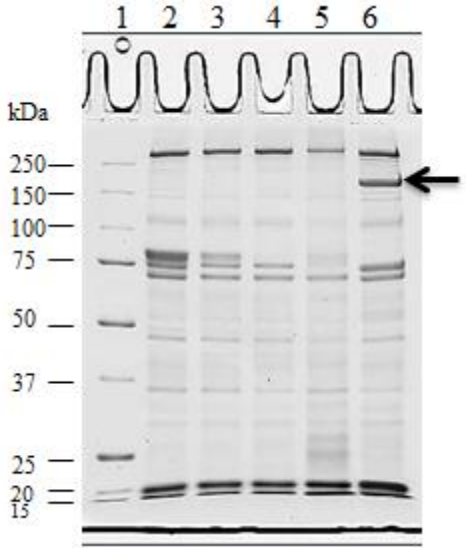


Fig. 8: The SDS-PAGE analysis of *P. apterus* female haemolymph monitoring the level of Vg on different days after the Pyrap-AKH treatment. Arrow indicates Vg. Lane 1 – MW markers; Lane 2 – day 1 without any treatment; Lane 3 – day 2 after treatment; Lane 4 – day 3 after treatment; Lane 5 – day 4 after treatment; Lane 6 – day 5 after treatment.

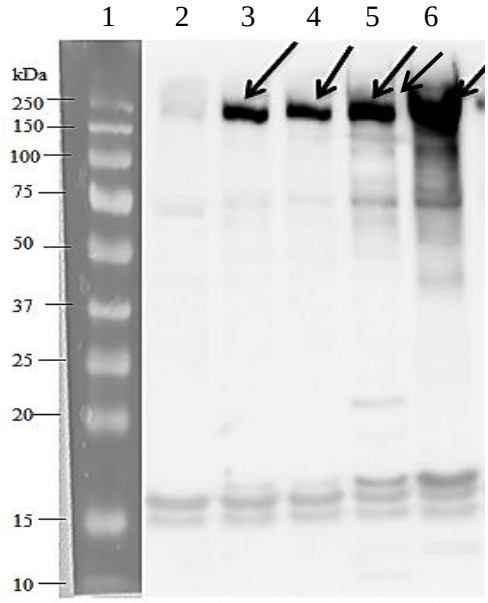


Fig. 9: Immunoblotting of the haemolymph proteins of *P. apterus* after SDS-PAGE on different days after the Ringer saline (control) treatment and after the reaction with the anti-Vg antibody. Vitellogenin is indicated with the arrows. For details see Material and Methods section. Lane 1 – MW markers; Lane 2 – day 1 without any treatment; Lane 3 – day 2 after treatment; Lane 4 – day 3 after treatment; Lane 5 – day 4 after treatment; Lane 6 – day 5 after treatment.

5 Discussion and Conclusion

Adipokinetic hormones play important role in the control of insect physiology and reproduction; this thesis was focused on their role in insect reproduction which was monitored by determination of the Vg level in the *P. apterus* female haemolymph. The relationship between two hormones – the intrinsic Pyrap-AKH and allatostatin III originally isolated from the cockroach *Diploptera punctata* (Woodhead et al. 1989) was investigated. Vitellogenin is synthesized by the fat body and it is the main protein stored in the oocyte. During female development, Vg level increases because its level is coordinated with developing oocytes. Both AKH and allatostatin terminate the Vg production in the fat body (Carlisle & Loughton, 1979; Kunkel & Nordin, 1985; Swevers et al., 2005). It is supposed that AKH inhibits Vg synthesis towards the end of the reproductive cycle when the terminal oocytes are large (Chapman, 1998). The allatostatin role in Vg inhibition seems to be indirect – this hormone blocks production of JH from CA and prevents its activities in the fat body cells. There, the JH influences vitellogenesis by binding to specific receptors in the cytosol, and controls directly the transcription of the Vg genes. It also regulates the Vg uptake into the

oocyte (Gäde, 1997). The injected allatostatin inhibits not only the JH but also ecdysteroid biosynthesis (Lorenz et al., 1997).

Accordingly, the Vg level in Pyrap-AKH and allatostatin injected fire-bug females was significantly reduced as compared with control females treated just by Ringer saline. In a similar experiment performed by Lorenz (2003), the ovaries of AKH-treated animals had a lower number of terminal oocytes which could be a result of the direct inhibition of egg maturation or the inhibition of protein and lipid synthesis in the female fat body. The main part of the organic substance found in insect eggs is the lipid, therefore, it is supposed that the inhibiting effect of AKH on the synthesis of lipid in fat body (Gokuldas et al., 1988) is an important reason for the impaired maturation of oocyte (Lorenz, 2003). Also the amount of proteins in the fat body was significantly lower after the AKH treatment because of the slow maturation of the oocytes and the reduced number of the terminal oocytes (Lorenz, 2003; Kodrík, 2008). It can be said that AKH has both the direct and indirect effect on the egg production; the direct effect is caused by inhibition of Vg synthesis while the indirect one by its inhibition of syntheses of other energy stores (lipids) in the fat body.

Vitellogenin is the main protein synthesized in the fat body and collected inside the oocyte through vitellogenesis (Atella et al., 2005). Following the SDS-PAGE, it was shown that the molecular weight of Vg of *P. apterus* is around 150 kDa. On day 2 after adult ecdysis, traces of Vg were found in the haemolymph and a gradual increase was observed in day 3, 4 and 5. The 150 kDa polypeptide becomes visible in the haemolymph with the commencement of vitellogenesis on day 2 after. This result coincides with earlier research reported by Socha et al. (1991). Although vitellogenesis is primarily controlled by JH and ecdysteroids, it can be said that AKH plays also important role in egg production (this study; Kodrík, 2008). The presence of allatostatin III in *P. apterus* females is also unfavourable to the occurrence of vitellogenesis indicating that the axis allatostatin-JH is critical for the Vg synthesis control in this species.. This is in accordance with results of Socha et al. (1991) who found that the application of JH analogue to allectomized *P. apterus* females restored the Vg production.

In summary, the study revealed that both the Pyrap-AKH and allatostatin III inhibit the presence of Vg in *P. apterus* female haemolymph, whereas the former hormone is significantly more effective than the latter one. It is supposed the Pyrap-AKH directly inhibits synthesis of Vg proteins, and maybe also lipids, in responsible cells of the fat body, while the allatostatin acts indirectly via curbing of JH production in corpora allata.

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Additional Resources

- ChemBioDraw Ultra software
- **Fig. 1** Retrieved on March 2, 2014 from: http://en.wikipedia.org/wiki/File:Bug_September_2008-3.jpg
- **Fig. 2** Retrieved on March 2, 2014 from: http://commons.wikimedia.org/wiki/File:Pyrrhocoris_apterus_group.jpg
- **Fig. 3** Retrieved on April 22, 2014 from: http://what-when-how.com/insects/juvenile-hormones-insects/

7 Supplement

Table 2: Quantification of Vg bands (using Relative Optical Densities) identified in *P. apterus* haemolymph samples at various times after the treatment of the control group with Ringer saline solution

Control	1	2	3	Average
1st day	52.5	42.6	45.1	46.7±5.1
2nd day	68.5	56.4	56.3	60.4±7.0
3rd day	1143.0	1088.9	1104.5	1112.1±27.8
4th day	2273.9	2003.7	2384.0	2220.5±195.6
5th day	3853.2	3606.2	3314.5	3591.3±269.6

Table 3: Quantification of vitelloganin bands (using Relative Optical Densities) identified in *P. apterus* haemolymph samples at various times after hormonal treatment with Pyrap-AKH

Pyrap-AKH	1	2	3	4	Average
1st day	47.9	37.5	68.5	35.3	47.3 ±15.1
2nd day	72.1	52.3	57.2	34.2	53.9±15.6
3rd day	79.6	58.8	62.0	53.7	63.5±11.2
4th day	88.8	74.8	69.9	83.5	79.3±8.4
5th day	568.2	473.1	510.2	453.9	501.3±50.3

Table 4: Quantification of vitelloganin bands (using Relative Optical Densities) identified in *P. apterus* haemolymph samples at various times after hormonal treatment with Allatostatin III

Allatostatin III	1	2	3	Average
1st day	52.2	60.6	61.6	58.1±5.1
2nd day	64.4	55.2	71.3	63.6±8.0
3rd day	133.2	102.7	127.3	121.0±16.7
4th day	806.9	858.5	759.8	808.4±49.3
5th day	2373.8	1852.6	2478.6	2235.0±335.2