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Master thesis

Endocrine regulation of oxidative stress in the red firebug *Pyrrhocoris apterus*

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Master thesis

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Annotation

A potential antioxidant effect of two insect hormones, Pyrap-AKH and 20-hydroxyecdysone, in the red firebug *Pyrrhocoris apterus* under conditions of oxidative stress was examined. Oxidative stress was induced by injection of herbicide Paraquat into the insect body. To verify that oxidative stress was developed sufficiently in the insects, thiobarbituric acid reactive substances, mainly malondialdehyde (TBARS) and protein carbonyls as principal biomarkers of oxidative stress were analyzed in CNS and hemolymph. A positive antioxidant effect of the two hormones was demonstrated by measuring content of GSH in brain and hemolymph, activity of γ-GTP in brain, and the total antioxidant activity in hemolymph.

I declare here that I wrote this work on my own, with usage of advices from my supervisor and of literature cited below.

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TABLE OF CONTENTS

Introduction **6-17**

A backgrounder on reactive oxygen species **6-7**

Formation and reactivity of main ROS **7-8**

Biological action of ROS **9-10**

Lipid peroxidation 9-10

Oxidative damage to proteins 10

Oxidative damage to DNA 10

Antioxidant defense mechanisms **11-13**

Superoxide dismutase 11

Glutathione peroxidase 11

γ-glutamyl transpeptidase 11-12

Glutathione 12

Catalase 12-13

Paraquat – a herbicide that generates free radicals **13**

Endocrine control of oxidative stress in insects **14-17**

20-hydroxy ecdysone – potential antioxidant I 14-15

Adipokinetic Hormone – potential antioxidant II 15-17

Materials and methods **18-23**

Experimental insects and chemical treatments **18**

Oxidative stress parameters **19**

Lipid peroxidation 19

Protein carbonylation 19

Extraction of the AKH from CNS and hemolymph **20**

Quantification of AKH by ELISA **20**

Antioxidant response in CNS and hemolymph **21-22**

Glutathione content 21

γ - glutamyl transpeptidase (γ-GTP) activity 21

Total antioxidant activity 21-22

Statistical analysis **22**

Results **23-25**

Dose response of PQ **23**
Experiments with 20E **23-24**
Oxidative stress biomarkers in membranes of brain tissue 23
Glutathione content and γ-glutamyl transpeptidase activity 23-24
Experiments with AKH **24-25**
AKH content in CNS and hemolymph in response to oxidative stress 24
Oxidative stress biomarker in hemolymph 24-25
Antioxidant response in hemolymph 25
Discussion **26-29**
The antioxidant properties of 20E **26-27**
The antioxidant properties of AKH **28-29**
General conclusion **30-31**
References **32-38**
Supplement 1. **39-48**
Supplement 2. **49**

INTRODUCTION

A backgrounder on reactive oxygen species

The discoveries of superoxide (O_2^-) by Gerschman et al. (1954) and superoxide dismutase by McCord and Fridovich (1969) opened the way for what is now a broad and far-reaching field of study involving the detection, characterization and analysis of the role of reactive oxygen species (ROS) in both normal and pathological processes of cellular metabolism.

ROS are formed during a variety of biochemical reactions and cellular functions. Most ROS come from the endogenous sources as by-products of normal and essential metabolic reactions, such as energy generation from mitochondria or the detoxification reactions involving the cytochrome P-450 enzyme system (Halliwell and Gutteridge, 1989). Exogenous sources include exposure to environmental pollutants, ionizing radiation etc

Oxygen breathing to produce energy involves generation of free radicals. During the whole mitochondrial oxidative phosphorylation and the respiratory chain, there is about 2% of the electron flow that may leak out from respiratory chain and reduce oxygen to O_2^-. When cells or tissues are under stressful or diseased conditions, the electron leakage may be greatly increased, thus causing more O_2^- formation (Beckman and Ames, 2000; Chance et al., 1979). Superoxide formation is the first step in a couple of reactions by which highly reactive oxygen derivates like hydrogen peroxide (H_2O_2) and hydroxyl radical ($OH^{\cdot}$) are produced.

When free radical formation exceeds the radical scavenging systems then a situation called “oxidative stress” is encountered and the effect of these toxic molecules on cell functions are collectively called “oxidative stress effects”. ROS generated within the organism or taken from their environment highly react with various organic molecules and impair diverse cell functions (Halliwell and Gutteridge, 1989). Inflicted tissue damage reduces performance and may threaten survival of the organism. ROS react with various cellular components including DNA, proteins, lipids/fatty acids and advanced glycation end products (e.g. carbonyls). These reactions between cellular components and ROS lead to DNA damage, mitochondrial malfunction, enzymatic inactivation, cell membrane damage and eventually cell death (apoptosis).

The cell has developed an elaborate antioxidant system, including various enzymes or molecules to scavenge intra- and extracellular ROS produced either in normal conditions or oxidative stress. These include superoxide dismutase (SOD), glutathione peroxidase (GPX),

catalase, the thioredoxin system, and nonenzymatic antioxidants and scavengers. However, it is highly likely that the endogenous antioxidant system may be overhelmed under conditions such as oxidative stress, leading to more ROS-induced cellular damage. By definition oxidative stress results when free radical formation is unbalanced in proportion to the protective antioxidants.

Formation and reactivity of main ROS

Atmospheric oxygen in its ground-state is distinctive among the gaseous elements because it is a biradical (it has two unpaired electrons). This feature makes oxygen very unlikely to participate in reactions with organic molecules unless it is "activated". A mechanism of activation is by the stepwise monovalent reduction of oxygen to form superoxide (O_2^-), hydrogen peroxide (H_2O_2), hydroxyl radical ($OH^{\cdot}$) and water molecule. The first three species are most common ROS.

Superoxide

The superoxide molecule (O_2^-) is a short-lived anion that is normally formed after one oxygen molecule accepts one electron from a reducing agent. O_2^- is an anion molecule that is impermeable to membranes such as the mitichondria inner membrane. However, anion channels have been shown to be able to facilitate O_2^- transport across the cell or mitochondrial membrane (Lynch and Fridovich, 1978; Vanden et al., 1998). O_2^- tends to spontaneously dismute to hydrogen peroxide, particularly when pH is about 4.7 (Bielski and Allen, 1977). O_2^- can also act as both reductant (E_0' for O_2^-/O_2 is about 0.33 V) and an oxidant (E_0' for H_2O_2/O_2^- is about 0.87 V) (Fee and Valentine, 1977), thus expanding its reactions with many biological molecules such as oxidation of both ascorbic acid and α-tocopherol (Nishikimi and Yagi, 1977) and reductions of both ketones (Frimer and Rosenthal, 1978) and metal cations (Patel, 1973). Here are some O_2^- related equations:

$$O_2 + e^- \rightarrow O_2^- \quad [1]$$

$$H_2O_2 \rightarrow HO_2^{\cdot} + e^- + H^+ \quad [2]$$

$$O_2^- + Fe^{3+} \rightarrow Fe^{2+} + O_2 \quad [3]$$

$$Fe^{2+} + H_2O_2 \rightarrow \mathbf{OH}^{\cdot} + OH^- + Fe^{3+} \quad [4]$$

$$O_2^- + H_2O_2 \rightarrow \mathbf{OH}^{\cdot} + OH^- + O_2 \quad [5]$$

Equation [1] and [2] describe the two possible mechanisms of O_2^- formation, either by oxygen reduction or H_2O_2 oxidation. Equations [3], [4] and [5] describe toxicity of O_2^- by generating $OH^{\cdot}$. O_2^- can also attack iron-sulfur clusters , resulting in the release of ferrous ion, which can bind DNA molecule before before its reaction with H_2O_2. Thus, catalyzed by the iron ion bound in DNA molecule, the $OH^{\cdot}$ would cause further damage and possible mutagenesis of DNA (Hassan, 1997).

Hydrogen peroxide

H_2O_2 is not a free radical since it has no unpaired electrons. However, it is one of the most important ROS. Compared to O_2^-, it is a stronger oxidant and is much more permeable to cell or mitochondrial membranes by passive diffusion. Here are some H_2O_2 related equations:

$$O_2^- + O_2^- + 2H^+ \rightarrow H_2O_2 + O_2 \quad [6]$$

$$O_2^- + e^- + 2H^+ \rightarrow H_2O_2 \quad [7]$$

$$O_2 + 2e^- + 2H^+ \rightarrow H_2O_2 \quad [8]$$

Compared to $OH^{\cdot}$, H_2O_2 has much less reactivity. The major toxicity of H_2O_2 arises from the interaction between H_2O_2 and iron known as Fenton reaction (described below).

Hydroxyl radical $OH^{\cdot}$

$OH^{\cdot}$ is a short-lived molecule (10^{-9} s in cell) and is impermeable to the membrane. However, it has a high reactivity and thus it can react with any molecules - it is a very strong oxidant and its attack to biological systems can cause extensive cellular oxidations (Buettner, 1993). $OH^{\cdot}$ can be generated by the Haber-Weiss reaction (equation [5]) or by Fenton reaction (equation [9]):

$$H_2O_2 + Fe^{2+}/Cu^+ \rightarrow OH^{\cdot} + OH^- + Fe^{3+}/Cu^{2+} \quad [9]$$

Biological action of ROS

Reactive oxygen species are able to cause an oxidative damage to lipids, proteins or nucleic acids. The mechanisms of these damages will be briefly described.

Lipid peroxidation

In cell membrane there are tremendous amounts of polyunsaturated fatty acids linked to the phospholipids. ROS like O_2^- or $OH\cdot$ can attack and oxidize these membrane lipids, resulting in loss of membrane integrity and function. For example, membrane fluidity, electrical resistance, membrane protein mobility, and the activity of ion pumps, can all be greatly reduced due to the loss of membrane lipids by ROS attack (Richter, 1987). The peroxidation of the lipids may be divided into 3 steps: initiation, propagation and termination. The initiation reaction between an unsaturated fatty acid and the hydroxyl radical involves the abstraction of an H atom from the lipid backbone forming the water molecule and carbon-centered lipid radical (equation [10]). In propagation phase, this lipid radical attacks molecular oxygen (biradical) and generates lipid peroxy-radical (equation [11]). This newly formed peroxy-radical can attack another fatty acid molecule, abstract a H atom and create lipid hydroxyperoxide and another carbon-centered lipid radical (equation [12]). Thus, one hydroxyl radical may start cycle of initiating and propagating reactions leading to massive lipid peroxidation in the cell. Lipid hydroxyperoxide may participate in a Fenton reaction in a presence of Fe leading to formation of reactive alkoxy-radical (equation [13]). This actually amplified the chain reaction caused by hydroxyl radical in the initiation step. The peroxidation reactions in membrane lipids are terminated when carbon or peroxy-radical cross-link to form conjugated, non-radical products (equation [14] – [16]). Normally, lipid peroxidation is measured by its own by-product such as malondialdehyde (MDA) or other thiobarbituric acid reactive substances (TBARS) and lipid hydroperoxides (LH)(Kukreja and Hess, 1994; Richter, 1987).

$$\text{Lipid-H} + OH\cdot \rightarrow H_2O + \text{Lipid}\cdot \qquad [10]$$

$$\text{Lipid}\cdot + O_2 \rightarrow \text{Lipid-OO}\cdot \qquad [11]$$

$$\text{Lipid-OO}\cdot + \text{Lipid-H} \rightarrow \text{Lipid-OOH} + \text{Lipid}\cdot \qquad [12]$$

$$\text{Lipid-OOH} + Fe^{2+} \rightarrow \text{Lipid-O}\cdot + OH^- + Fe^{3+} \qquad [13]$$

$$\text{Lipid}\cdot + \text{Lipid}\cdot \rightarrow \text{Lipid-Lipid} \qquad [14]$$

$$\text{Lipid}\cdot + \text{Lipid-OO}\cdot \rightarrow \text{Lipid-OO-Lipid} \quad [15]$$

$$\text{Lipid-OO}\cdot + \text{Lipid-OO}\cdot \rightarrow \text{Lipid-OO-Lipid} + O_2 \quad [16]$$

Oxidative damage to proteins

Oxidative attack on proteins results in site-specific amino acid modifications, fragmentation of the peptide chain, altered electrical charge and increased susceptibility to proteolysis. Primary, secondary, and tertiary protein structures alter the relative susceptibility of certain amino acids. Oxidative modifications of protein molecules include reversible oxidation of sulfur containing aminoacids, leading to form disulphide bridges (cysteine residues) or sulphoxide derivates (methionine residues). Alternatively, a site-specific oxidation of arginine, lysine and histidine leads to irreversible carbonyl groups formation. However, there is much less possibility of chain reaction in protein oxidation compared to lipid peroxidation. If the amino acid modified by ROS is the key residue for the enzymatic function, the influence of such a minor modification will be tremendous. Also some enzymes with antioxidant activities, such as glutathione peroxidase and catalase, can be inactivated by ROS under proper conditions, resulting in possible imbalance between ROS and antioxidant defense system (Fridovich and Freeman, 1986). Fortunately, in normal cells, most modifications of amino acid residues are no-specific, leading to much less cellular damage.

Oxidative damage to DNA

Activated oxygen and endogenous ROS can attack DNA to generate lesions causing deletions, mutations and other lethal genetic effects. Characteristic of this damage to DNA has indicated that both the sugar and the base moieties are susceptible to oxidation, causing base degradation, single strand breakage, and cross-linking to protein (Imlay and Linn, 1986). For example, 8-hydroxy guanine (8-OH-Gua) is one of the most common lesions caused by ROS attack. The formation of 8-OH-Gua may lead to erroneous GC→TA transversions during DNA replication, resulting in possible mutagenesis. Other modified bases by ROS include 5-hydroxyuracil, 5-hydroxycytosine, and 8-hydroxyadenine, which all have premutagenic function (Wallace, 1998).

Antioxidant defense mechanisms

Superoxid dismutase (SOD)

SODs are metalloenzymes that catalyze the dismutation of O_2^- to H_2O_2 (equation [17]):

$$2O_2^{\bullet -} + 2H^+ \rightarrow H_2O_2 + O_2 \qquad [17]$$

SODs is a 32-150kD dimeric or tetrameric enzyme which can be found in all oxygen-consuming organisms and can be divided into two major families based on the metal located at the active site: the copper-zinc SODs (Cu,ZnSODs) and the iron/manganese SODs (Fe/MnSODs). These dismutases are mainly distributed in prokaryotic or eukaryotic cells. However, there is also existence of extracellular Cu,ZnSODs, a secretory glycoprotein located in plasma and tissues (Beckman and Ames, 2000; Suzuki et al., 2000). Lower concentrations of SOD have protective effect on cells by ameliorating ROS-induced injuries whereas too high concentrations may not benefit tissue due to the "overscavenging effect".

Glutathion peroxidase (GPX)

The selenium-dependent GPX is an 85kD tetramer, whose function is to convert H_2O_2 to water using the hydrogen atoms from reduced glutathione (GSH) forming oxidized glutathione (GSSG) (equation [18]):

$$2GSH + H_2O_2 \rightarrow GSSG + 2H_2O \qquad [18]$$

GPX can utilize various organic peroxides and some lipid peroxides to convert GSH to GSSG.

γ-glutamyl transpeptidase (γ-GTP)

γ-glutamyl transpeptidase catalyses transfer of γ-glutamyl group from glutathione or other γ-glutamyl compounds to amino acids or dipeptides , and also hydrolyses the γ-glutamyl bond. This enzyme, which is also known as γ-glutamyl transferase, plays an important role in glutathione metabolism because the reactions catalyzed by the enzyme are the first steps in the degradation of glutathione, which involves the cleavage of the bond between glutamate and cysteine and the transfer of the glutamate residues to an acceptor amino acid (equation [19]). Subsequently the Cys-Gly dipeptide is degraded by dipeptidase (equation [20]) and Glu-aa by

glutamylcyclotransferase (equation [21]). This is an ectopic enzyme which enables extracellular glutathione to be transported into the cell.

$$\text{Glu-Cys-Gly} + \text{aa} \rightarrow \text{Glu-aa} + \text{Cys-Gly} \quad [19]$$

$$\text{Cys-Gly} \rightarrow \text{Cys} + \text{Gly} \quad [20]$$

$$\text{Glu-aa} \rightarrow \text{5-oxoproline} + \text{aa} \quad [21]$$

Glutathione (GSH)

Glutathione is a tripeptide (Glu-Cys-Gly) whose antioxidant function is facilitated by the sulphhydryl group of cysteine (Rennenberg, 1982). GSH is one of the most important antioxidants in cells, being used in enzymatic reactions to eliminate peroxides and in non-enzymatic reactions to maintain ascorbate and α-tocoferol in their reduced and functional forms. It can also react chemically with superoxide and hydroxyl radicals and therefore function directly as a free radical scavenger.

The synthesis and degradation of GSH occurs continuously through the glutamyl cycle that has been well characterized in animals (Meister, 1988). The first step in GSH synthesis is the combination of glutamate and cysteine to form glutamylcysteine by the enzyme glutatamylcysteine synthetase (equation [22]). The subsequent step involves the addition of glycine by the enzyme glutathione synthetase (equation [23]).

$$\text{Glu} + \text{Cys} \rightarrow \text{Glu-Cys} \quad [22]$$

$$\text{Glu-Cys} + \text{Gly} \rightarrow \text{Glu-Cys-Gly} \quad [23]$$

The reduction of GSSG to GSH is catalysed by the enzyme glutathione reduktase (GR).

Catalase

Catalase is a 24kD homotetrameric enzyme with a heme-iron active center. The main function of this enzyme is to decompose toxic H_2O_2 into water and oxygen (equation [24]):

$$2H_2O_2 \rightarrow O_2 + 2H_2O \quad [24]$$

Catalase is mainly located in peroxisomes, one of the major sites for intracellular H_2O_2 formation. Compared to GPX, catalase tends to reduce small peroxides such as H_2O_2, but has

no effect on larger molecules such as lipid hydroperoxides. Catalase is low-affinity but high-capacity enzyme, perfectly suited for H_2O_2 scavenging in peroxisomes or mitochondria where higher level of ROS can be produced. On the other hand, GPX is a high-affinity but low-capacity enzyme well distributed in cells, and can work on peroxides with large molecular weights (Warner and Wispe, 1997).

Paraquat – a herbicide that generates free radicals

Paraquat is a nonvolatile, ionic compound used as a herbicide that is almost completely insoluble in organic solvents, which is typical for the bipyridyl group of chemicals (picture 4.). Application of Paraquat causes apparent increase of ROS not only in plants but also in living organisms and that is the reason why is PQ frequently employed in investigation on the oxidative stress management in invertebrates, especially in insect.

The biochemical mechanism of paraquat toxicity is related to the cyclic oxidation and reduction of paraquat, which leads to continued production of high levels of superoxide anion (O_2^-) and other cytotoxic oxygen free radicals. Superoxide anion and other oxygen free radicals initiate the peroxidation of membrane lipids, causing tissue damage and death. Paraquat oxidation is coupled with the reduction of molecular oxygen, forming superoxide anion, singlet oxygen, and hydroxyl radicals. These molecular species react with polyunsaturated fatty acid free radicals and, on further oxidation, with lipid hydroperoxide radicals. The hydroperoxide radicals then maintain the formation of new fatty acid radicals while being converted to lipid peroxides in a chain reaction. Various enzymes in the cells catabolize the superoxide radical and reduce the lipid hydroperoxides to less-toxic lipid alcohols. The superoxide anions are converted to hydrogen peroxide and oxygen; hydrogen peroxide is further inactivated to water and oxygen by catalases and peroxidases. In the presence of reduced nicotinamide adenine dinucleotide phosphate (NADPH), paraquat is reduced by microsomal NADPH-cytochrome reductase. The reduction of lipid peroxides by glutathione peroxidase requires reduced glutathione. Because the reduction of oxidized glutathione is coupled with NADPH oxidation by way of glutathione reductase, it seems that the availability of NADPH is essential for paraquat detoxification, and that the critical depletion of NADPH may render the cell more susceptible to lipid peroxidation.

Endocrine control of oxidative stress in insects

20-hydroxy ecdysone – potential antioxidant I

Ecdysteroids (ES) are the generic name for a group of related steroid hormones (picture 1.) that are primarily involved in the molting process of arthropods but also have wide-ranging effects in every developmental stage. The group of ES in *P. apterus* includes makisterone A and ecdysone, a precursor of 20-hydroxyecdysone (20E). 20E is the true molting hormone in that it is most active in inducing a molt. The precursors for ES synthesis by the prothoracic gland (major source of ES) of insects are sterols, such as cholesterol. Zoophagus insects can easily ingest sufficient cholesterol in food. Phytophagus insects have to utilize several sterols from plants to form cholesterol. Synthesis of ES by the prothoracic gland of an insect larva occurs by the action of PTTH (picture 2.). Synthesis of ES during other developmental stages by additional sources such as ovaries and testes occurs in response to other ecdysiotropic neurohormones (ovarian ecdysiotropic hormones and testes ecdysiotropin).

The prothoracic gland is not the only source of ES. Even though the prothoracic gland degenerates in adult insects, ES still occur in their hemolymph. In these adults, the site of ES synthesis has been shifted to the ovaries and the testes. In many female insects, ES are produced by the follicle cells of the ovaries, where they are conjugated to other molecules and incorporated into the eggs for later use during embryogenesis.

ES, as typical steroid hormones, can easily diffuse into cells. They directly affect gene expression, causing the activation or inactivation of certain genes and the resulting synthesis or inhibition of enzymes and other regulatory peptides. Although all tissues of the insect can potentially be exposed to the ES that are circulating in the blood, not all cells respond in the same way. Different ES receptor isoforms have been identified in different cells that show different responses to ES during metamorphosis.

Estrogens were shown to inhibit the oxidation of cholesterol and the peroxidation of polyunsaturated fatty acids (diene conjugation or malondialdehyde formation) in low density lipoproteins (LDL), microsomes, and other components of biological systems (Lacort et al., 1995; Taniguchi et al., 1994). The estrogens and their catechol or methyl ether metabolites resemble hindered phenols such as t-butyl hydroxyanisol or t-butyl hydroxytoluene that are commonly used antioxidants. The phenyl ring is essential for the antioxidant action that rests on the formation of intermediate phenoxy radicals, which are subsequently reduced back to phenols by other cellular antioxidants (Subbiah et al., 1993; McHugh et al., 1998; Liehr and Roy, 1998).

Based on standard assays with mammalian cells, antioxidant activity was also demonstrated for ecdysteroids that do not contain a phenyl ring (Cai et al., 2002). The goal of the second part of this work was to verify antioxidant properties of 20-hydroxyecdysone (20E) and to explore whether it may protect insect tissues against the oxidative stress. Investigations were performed with adult firebug, *Pyrrhocoris apterus*. Brain was used in most tests because, in contrast to other organs such as gut, fat body, and gonads, it does not change in size during the reproductive cycles and in dependence on age. Action of oxidative radicals on the neural tissues has been documented in mammals; a number of neurodegenerative diseases are actually caused or accentuated by oxidative radicals that elicit apoptosis in the neural cells (Klein and Ackerman, 2003).

PQ reacts with oxygen in a redox cycle composed of several electron transfer reactions that produce superoxide, singlet oxygen, hydroxyls, hydrogen peroxide, lipid peroxides, and disulfides (Bus et al., 1974, 1976; Hassan and Fridovich, 1979). Lipid peroxidation and the depletion of glutathione (GSH) by the flux of oxidative radicals were reported to be the main cause of paraquat toxicity (Shu et al., 1979; Trush et al., 1981 Kelner and Bagnell, 1990). We have assessed the level of oxidative stress from the measurements of lipid peroxidation, protein carbonyl formation, membrane microviscosity and the concentration of GSH. The reduction of GSH was correlated with the activity of γ-glutamyl transpeptidase, an enzyme important in GSH recycling (Karp et al., 2001).

To check whether the antioxidative action of 20E protects the whole organism against the damaging effects of paraquat, monitoring the hemolymph protein profile and the female reproduction potential was included in the study (results shown in attached papers). Hemolymph proteins reflect metabolic activities of the gut, fat body, hemocytes, and gonads, and to lesser extent of other organs. The changes in reproduction indicate alterations in the supply of nutrients and/or in hormonal regulations. The hemolymph proteins and the reproduction parameters taken together provide a good indication of the overall effects of tested compounds.

Adipokinetic Hormone – potential antioxidant II

Insect metabolism and especially the part related to generation of energy is controlled by adipokinetic hormones (AKHs), small peptides compound mostly of 8 to 10 amino acids, which are synthesised, stored and released by neurosecretory cells from the corpora cardiaca (CC), a neuroendocrine gland connected with the insect brain. The hormones have been isolated from

representatives of many insect orders (Gäde et al., 1997; Gäde & Goldsworthy, 2003). Generally, they behave as typical stress hormones – they stimulate catabolic reactions (mobilise lipids, carbohydrates and/or certain amino acids), making energy more available, while inhibiting synthetic reactions. Using this strategy they direct entire energy to combat the immediate stress problems and suppress processes that are momentarily unimportant or even those that could draw on the mobilized energy.

Majority of AKHs are synthesised in glandular cells and stored in storage lobus of the CC - from this organ they are released into the haemolymph, but the brain of some insects also contains AKH-like material. A physiological role of the AKH in the brain may be associated with a neuromodulatory role (Milde et al., 1995). Also a role in increasing of intracellular calcium level in neurons (Wicher et al., 1994) can not be excluded.

The main target tissue for AKH action is the fat body (picture 3.). AKH as a peptidic hormone is not able to freely penetrate through the cell membrane into the cytoplasm, hence the transduction is mediated via AKH receptor (Park et al., 2002; Staubli et al., 2002; Kaufmann & Brown, 2006; Hansen et al., 2006). The corresponding G-protein activates 2 (or 3) biochemical pathways that lead to production of simple oxidable products like trehalose, diacylglycerol (DAG) or proline.

Due to multifunctional and pleiotropic function, AKHs are involved in a large number of well-described actions such as cardiostimulation, inhibition of the synthesis of RNA, fatty acids and proteins in the fat body, stimulation of biosynthesis of mitochondrial cytoheme a + b, including the induction of gene expression for a cytochrome P-450 enzyme, and stimulation of oxidation of substrates by the flight muscles (Gäde et al., 1997; Gäde, 2004). AKHs also function as stress responsive hormones to insecticide treatment (Samaranayaka, 1974; Candy, 2002; Kodrík and Socha, 2005) photophase interruption (Kodrík et al., 2005) as well as infection by pathogens (Goldsworthy et al., 2002, 2003a,b; Mullen and Goldsworthy, 2003; Goldsworthy et al., 2005). Recently, a multiple increase of AKH content in CC and hemolymph of the Colorado potato beetle *Leptinotarsa decemlineata* when fed genetically modified potatoes expressing *Bacillus thuringiensis* and *Galanthus nivalis* toxins concomitant with oxidative stress in gut tissues presumably because of survival challenge was demonstrated (Kodrík et al., 2007). The results were mimicked by the application of Paraquat, a bypyridilium herbicide that creates conditions of oxidative stress (OS) by undergoing cyclic redox reactions with oxygen during microsomal and electron transfer reactions (Hassan, 1984).

This thesis work is linked to the previous study on *L. decemlineata* (Kodrík et al., 2007) and I have examined the AKH antioxidant characteristics and determined if AKH modulation of

OS is more general in insects. To investigate this phenomenon I tested the effect of Paraquat on the firebug, *Pyrrhocoris apterus* (L.). This bug possesses two adipokinetic peptides – Pyrap-AKH (Kodrík et al., 2000) and Peram-CAH-II (Kodrík et al., 2002b) - and is a very convenient model insect for this type of studies thanks to detailed information available about its biology (Socha, 1993). Here, I have attempted to test the following hypothesis: (a) whether AKH is increased in CC and hemolymph upon oxidative stress, and (b) if such an increase potentiates an antioxidant response in the insect body, particularly the hemolymph.

This work was compiled from two papers, which are “in press” in scientific journals and are attached at the end of this work in SUPPLEMENT 2.

MATERIALS AND METHODS

Experimental insects and chemical treatments

The stock culture of the bug *Pyrrhocoris apterus* (Heteroptera) originated from a wild brachypterous population collected at České Budějovice (Czech Republic, 49°N). All stages from egg to adult were kept in half-liter glass jars supplied with linden seeds and water. Insects were reared at 26 ± 1°C under long-day conditions (LD 18:6h).

For experiments with 20E, males and females were kept separately since the first day after the adult ecdysis and used for the experiments four days later when the males reached a body weight of 29.6 ± 1.8 mg and the females weighed 36.9 ± 1.3 mg. The insects were then injected individually with 38 pmol Paraquat (PQ, 1,1'-dimethyl-4,4'-bipyridilium dichloride hydrate), 1 pmol 20-hydroxyecdysone (20E), and with both PQ and 20E, respectively. These amounts proved non-toxic but effective in respect to oxidative stress in preliminary experiments (data not shown). PQ was dissolved in insect saline and 20E in 10% propanol (10 mM stock) followed by dilution in the saline. Insects were injected with 2 µl of appropriate solution(s) that was dispersed from a 10 µl syringe (Hamilton, Bonaduz, Switzerland). Insects injected with 2 µl saline served as controls.

For AKH experiments, freshly ecdysed brachypterous adult males were transferred to fresh jars and used for experiments when they were approximately 10 days old. Preliminary experiments revealed that males are more responsive to oxidative stress, while females are comparatively more tolerant. Furthermore, when females are in the reproductive phase, the results tend to get a bit varied. Hence, for this study, we chose only males. The insects were individually injected with 2 µl solution containing 40 pmol Paraquat or with 2 µl Ringer saline which served as controls, and CNS and hemolymph was sampled at 4 hours post inoculation (hpi). This dose was fixed after a dose response study (PQ vs. oxidative stress biomarker), and the 4 hpi was taken as standard since distinct changes in both protein carbonylation and AKH titre were recorded at this time. In certain experiments, the bug's AKH (Pyrap-AKH) was injected alone (40 pmol) to some individuals or in combination with PQ (1:1, 40 pmol each) (Večeřa et al., 2007).

All chemicals used in experiments with both 20E and AKH were obtained from Sigma (St. Louis, MO, USA) except for 20E (98% purity) which was extracted from the plant *Rhaponticum carthamoides* by our colleague Dr. P. Šimek.

Oxidative stress parameters

Lipid peroxidation

Thiobarbituric acid (TBA) reactive substances (TBARS) were quantified as a marker of lipid peroxidation (Uchiyama and Mihara, 1978). CNS were mixed (1:5, v:v) with phosphoric acid (0.2%, v:v) and homogenized, than heated with TBA (0.6% final concentration, stabilized with 1 mM butylated hydroxytoluene in 50 mM NaOH) to 95°C for 30 min. Cooled mixture was partitioned with 1-butanol and TBARS were obtained from the butanol phase after solvent evaporation in a vacuum centrifuge. The residues were reconstituted in elution buffer (35% methanol in 50 mM KH_2PO_4-KOH, pH 7.0) and applied on the analytical reverse phase C18 HPLC column (Purospher RP-18, 5µm: LiChroCART 250-4, E-Merck, Darmstadt, Germany). The sample was run under isocratic conditions at a flow rate 0.5 ml/min in the Automated Waters^{TM} HPLC system (515 Pump, 2475 multi λ fluorescence detector) driven by a DataApex Clarity software (Ver.2.3.0.195; Prague, Czech Republic). The peak of fluorescence (λ_{Ex} 515 nm and λ_{Em} 553 nm) corresponding to the melondialdehyde (MDA) adduct MDA-$(TBA)_2$ occurred at RT = 27.93. The adduct was prepared in standard way using 1,1,3,3 tetramethoxypropane as the starting reagent. The recovery of membrane TBARS varied between 92-96% when some of the samples were spiked with known concentrations of MDA. The results were expressed as MDA equivalents in nmol.mg^{-1} protein.

Protein carbonylation

Carbonyls were quantified after their reaction with 2, 4-dinitrophenylhydrazine (DNPH) (Levine et al., 1990). Samples (homogenized CNS or hemolymph) were treated with 7 mM DNPH (sample) or 2 M HCl (control). After incubation in dark at room temperature for 1 h, proteins were precipitated with 28% trichloroacetic acid, and centrifuged at 10,000g. The pellet was re-suspended in 5% trichloroacetic acid on ice and centrifuged again. Precipitated proteins were re-suspended manually, washed twice with ethanol/ethylacetate (1:1), and dissolved in 6 M guanidine hydrochloride. The carbonyls were quantified at 370 nm in the SpectraMax ELISA microtitre plate reader (Molecular Devices, Sunnyvale CA, USA). Results were expressed as nmol.mg^{-1} protein using the extinction coefficient 22,000 M^{-1} cm^{-1}. Bovine serum albumin standard curve was used in the absorption measurements (280 nm) of protein concentrations in the guanidine solutions. Protein carbonyl values were corrected for interfering substances by subtracting the A_{370}/mg protein measured without DNPH (controls).

Extraction of the AKH from CNS and hemolymph

Methanolic extract (80% methanol) of the CNS (brain with the CC and corpora allata attached) was used for determination of the AKH content in the CNS by means of a competitive ELISA (see below). For determination of the endogenous AKH in the hemolymph by the ELISA, some pre-purification steps were essential (Goldsworthy et al., 2002). The cumulative hemolymph samples collected always from 8 – 10 males were extracted in 80% methanol and after centrifugation the supernatants of these extracts were evaporated to dryness. Afterwards, the solid phase extraction (Sep Pak C18, Waters) and HPLC pre-purification were employed. Briefly, fractions eluting in 60% acetonitrile (Merck, Darmstad, Germany) from the Sep Pak column were evaporated again and taken for HPLC analysis on the Waters chromatography system, at a flow rate 2 ml/min and fluorometric detection at λ_{Ex} = 280 nm and λ_{Em} = 348 nm. The samples were fractionated on the Chromolith Performance RP-18e column (Merck) 100 x 4.6 mm with gradient 0-2 min 25% B, 2-12 min 25-87% B and 12-15 min 87% B (A = 0.11% trifluoroacetic acid (TFA) in water, B = 60% acetonitrile in 0.1% TFA). Fractions eluted between 6.0 – 8.0 min were then taken for the ELISA competitive tests (retention times of the synthetic Pyrap-AKH and Peram-CAH-II are 6.87 and 7.03 min, respectively, in this system, Kodrík et al., 2002b; 2003). The efficiency of recovery of hemolymph AKH during the extraction procedure was checked by adding 500 fmol of Pyrap-AKH to 20µl samples of hemolymph before the extraction. The recovery of AKH (74.8 ± 8.2 %; mean ± SE) was checked using ELISA (see below) and estimated from five separate parallel measurements; all respective data were corrected for losses incurred.

Quantification of AKH by ELISA

A competitive ELISA was used for determination of the AKH content in *P. apterus* CNS (1/4 CNS equiv.) and hemolymph (10 µl equiv.) according to our protocol described earlier (Goldsworthy et al., 2002; Kodrík et al., 2003). Rabbit antibodies were raised commercially against Cys^1-Pyrap-AKH (Sigma Genosys Biotechnologies Ltd) and the resulting antibody recognized both the Pyrap-AKH and Peram-CAH-II. The ELISA comprised precoating of the 96-well microtitre plates (high binding Costar, Corning Incorporated, Corning, New York) overnight with IgG dilution 1:10000 for CNS and 1:2000 for hemolymph. A biotinylated probe prepared from Cys^1-Pyrap-AKH using Biotin Long Arm Maleimide (BLAM, Vector Laboratories, UK) was used in the assay (for details see Kodrík et al., 2003). The Pyrap-AKH (Polypeptide Laboratories, Praha) was used as a standard for AKH quantification.

Antioxidant response in CNS and hemolymph

Glutathione content

Glutathione (GSH) and its oxidized product GSSG were quantified according to Griffith (1980) with some modifications (Krishnan et al., 2007b). Hemolymph was diluted 8x in potassium phosphate buffer (50 mM, pH 7.8) containing Na – EDTA (6.3 mM), and centrifuged (10,000 g for 10 min at 4°C); in the case of brains, 20 CNS were homogenized and processed in the same way. The supernatants were mixed in microtitre wells with 0.2 mM NADPH and glutathione reductase (0.01 U/ml) and incubated at 37°C for 10 min before 6 mM 5,5'-dithiobis(2-nitrobenzoic acid) was added. The change of absorbance at 412 nm was recorded for 30 min in 5 min intervals in a Spectramax microplate reader. Total glutathione (GSH + GSSG) was quantified against a calibration curve of GSH (0 to 100 µM) using the kinetic method (slope) and expressed as nmol.mg^{-1} protein. GSSG was measured separately after its derivatization with 2-vinyl pyridine (incubation at 30°C for 1 h) added to sample aliquots. The amount of GSSG was read from the calibration curve of the GSSG standard (0 to 100 µM) using the kinetic method (slope) and expressed as nmol. mg^{-1} protein. The amounts of reduced GSH were calculated by subtracting the content of GSSG from that of the total glutathione (GSH+GSSG).

γ- glutamyl transpeptidase (γ-GTP) aktivity

γ-GTP was assayed with a modified procedure of Ikeda (2000). Tissue (20 brains) were homogenised in 500 µl of 10 mM Tris-HCl, pH 7.8, in ultrasonic homogenizer 4710 (Cole Parmer, Chicago, IL, USA) at 40-50 MHz in 20 s bursts for 2 min on ice. The homogenates were centrifuged (25,250g, 4°C) and 10 µl of supernatant was added to microwells containing 200 µl 1 mM L-γ-glutamyl-p-nitroanilide and 20 mM glycylglycine in 0.1 M Tris-HCl (pH 8.0). The increase of absorbance at 410 nm was recorded at 37ºC in 1 min intervals for 15 min in the SpectraMax ELISA microtitre plate reader. The enzyme activity was expressed as U.mg^{-1} protein using 8.8 $mM^{-1}cm^{-1}$ as the molar extinction coefficient of p-nitroaniline.

Total antioxidant aktivity

Total antioxidant activity (TAA) was estimated according to Rice-Evans and Miller (1994) with some modifications for a microtitre plate assay. The formation of ferrylmyoglobin radicals from metmyoglobin (MetMb) and hydrogen peroxide in the presence of the peroxidase substrate 2,2´-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) produces the ABTS

radical cation ($ABTS^{\bullet+}$), and an intense blue/green chromogen with characteristic absorption maxima at 734 nm. The formation of this colored radical cation is suppressed by the presence of hydrogen donating antioxidants and the extent of this suppression is directly related to the antioxidant activity of the sample being investigated. We used a spectrophotometric microtitre plate assay to determine the TAA of hemolymph samples from experimental groups and a reference antioxidant, the water soluble analog of Vitamin E, Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid). Briefly, 5x diluted hemolymph was mixed with 70 µM metmyoglobin (from horse heart) and 2.5 mM ABTS. The reaction was initiated by addition of 1 mM of hydrogen peroxide and taking kinetic readings over a period of 5-7 minutes or end point reading at 5 minutes at 27°C. The kinetics of the quenching of the ABTS radical cation was followed at 734 nm using a Spectramax microplate reader with a temperature control. TAA values of samples were calculated from a Trolox standard curve that showed a linear relationship between concentration (0-2 mM) and the length of the lag phase before the production of the ABTS radical cation.

Statistical analysis

Statistical analysis of both AKH and 20E experiments was performed and the graphs generated with GraphPad Prism 4 (GraphPad Software Inc., San Deigo, CA, USA).). For 20E experiments, two-way ANOVA with Bonferroni post-hoc tests were used to compare male and female responses to a treatment and one-way ANOVA with Dunns multiple comparisons were applied to test significance of the effects of different treatments on a single parameter.

In experiments with AKH, each assay was done on samples collected from 8-10 individuals. The results in the graphs represent the mean of measurement ± SE (n = 7-10). Unpaired t-test was used to evaluate statistical significance in Fig. 1 and in other cases (Figs 5, 6 and 7) one-way ANOVA with Tukey's multiple comparisons were applied to test significance of the effects of different treatments on a single parameter.

Kaplan-Meier survival analysis was conducted to test survival proportion and the median survival time after different treatments. Survival curves were compared using the logrank test.

RESULTS

Dose response of PQ

PQ showed a dose dependent increase in protein carbonyl levels (Fig. 1). PQ was prepared in concentrations ranging from 2.5 to 120 µM and injected in insects between the range 5 to 240 pmoles. At 40 pmol we observed a distinct elevation in protein carbonyl levels at 4 hours post inoculation. Insects injected with this dose survived more than 5 days, while higher doses (80 to 240 pmol) resulted in cytotoxicity and enhanced mortality. Hence for all subsequent experiments we chose 40 pmol as the standard "physiological" dose.

Experiments with 20E

Oxidative stress biomarkers in membranes of brain tissue

The amount of thiobarbituric acid reactive substances (TBARS) was significantly enhanced ($p < 0.0001$) in the brain microsomal fraction from males (5 fold) and females (3 fold) injected with PQ (Fig. 2a), indicating considerable peroxidation of membrane lipids. TBARS content in insects treated with 20E was similar to the sham-injected insects. However, 20E injection significantly ($p<0.0001$) allayed the TBARS increase caused by PQ. It is noticeable that the stimulation of TBARS content by PQ was distinctly ($p<0.001$) higher in the male than in the female brain membrane fractions. This sex-related difference persisted ($p<0.05$) when the stress was mitigated with 20E. While in the males the reduced level of TBARS was still higher ($p<0.001$, 2 fold) than in the controls, 20E injection in the females suppressed TBARS to a concentration statistically indistinguishable from the controls.

PQ application also elevated the content of protein carbonyls in the microsomal membranes of the male (6 fold, $p<0.0001$) and female brains (4 fold, $p<0.0001$) (Fig. 2b). A co-injection of 20E suppressed the PQ-induced enhancement 1.6 fold in males ($p<0.01$) and 2 fold in the females ($p<0.0001$). Carbonyl content in the PQ injected insects was consistently higher in males than in females; the sex difference was particularly conspicuous ($p<0.01$) in insects receiving PQ simultaneously with 20E.

Glutathione content and γ-glutamyl transpeptidase activity

The content of reduced glutathione (GSH) was markedly depleted in both male (4 fold, $p<0.0001$) and female (3 fold, $p<0.0001$) brains following PQ injection (Figure 3a). This decrease was much less severe in males and virtually abolished in the females when 20E was

administered concurrently. The difference between GSH levels in the PQ-injected and PQ+20E-injected insects was 2 fold in males ($p<0.0001$) and 2.5 fold in females ($p<0.0001$). No significant differences in the GSH content were recorded between brains of the control (sham-injected) and 20E-injected (without PQ) insect groups. Female brains exhibited consistently higher GSH levels than the male brains; the difference was significant in the insects injected with PQ ($p<0.01$) and even more in those injected with PQ and 20E ($p<0.0001$).

Brain activity of membrane γ-GTP (an enzyme whose active site is extracellular), which plays a prime role in the GSH metabolism, was reduced after the PQ injection 2 fold in males ($p<0.005$) and 1.8 fold in the females ($p<0.005$) (Figure 3b). 20E treatment did not significantly alter the control enzyme activity but alleviated the suppressive PQ action: activity measured after the treatments with both PQ and 20E was significantly higher than after the PQ injection alone in both males ($p<0.03$) and females ($p<0.001$). In females, the rescued values were practically identical with the controls (non significant) but in the males they remained distinctly lower than in the untreated controls ($p<0.03$) or in insects receiving just 20E. The female brains always harbored higher γ-GTP activity than the male brains; the difference was most obvious ($p<0.003$) after the concurrent PQ and 20E injection.

Experiments with AKH

AKH content in CNS and hemolymph in response to oxidative stress

Total AKH content in CNS (Pyrap-AKH plus Peram-CAH-II) was not altered significantly upon oxidative stress created as a result of PQ injection (40 pmol), however, in hemolymph a significant and multiple increase of AKH titer was recorded (ca. 5 fold, $p<0.05$) at 4 hours post inoculation compared with Ringer saline (control) alone injected individuals (Fig. 4a and b). Intact insects did not show any significant changes either in CNS or in hemolymph when compared with Ringer saline injected individuals (data not shown).

Oxidative stress biomarker in hemolymph

Injection of 40 pmol PQ to *P. apterus* males resulted in a significant (ca. 2 fold, $p<0.01$) enhancement of protein carbonyls (a specific biomarker of oxidative stress) in hemolymph at 4 hpi compared with those injected with Ringer saline (controls), AKH (Pyrap-AKH, 40 pmol) and/or AKH:PQ (40 pmol each) (Fig. 5). AKH injection alone did not result in any significant changes in the protein carbonyl content of hemolymph. Interestingly co-injection of AKH with PQ brought down the protein carbonyl levels to almost identical levels as in case of control or

AKH alone injected individuals. Intact insects again did not show any significant changes in carbonyl contents compared with Ringer alone injected individuals (data not shown).

Antioxidant response in hemolymph

We chose to measure the antioxidant response in hemolymph since it was the first tissue that encountered oxidative stress conditions upon injection of PQ where we recorded changes in AKH. Two specific parameters were taken for assessment of the antioxidant response: the reduced GSH content and the total antioxidant activity of cell free hemolymph.

The GSH content in hemolymph was significantly depressed (ca. 2.7 fold, $p<0.05$) when PQ was injected in *P. apterus* male compared with controls (Fig. 6). On the other hand, AKH (Pyrap-AKH-I, 40 pmol) injection alone caused a significant and multiple (ca. 2 fold, $p<0.001$) increase in GSH content compared with controls. Co-injection of AKH with PQ resulted in enhanced (ca. 2.4 fold, $p<0.05$) GSH content compared with PQ-injected individuals, however, this level was significantly less (ca. 2 fold, $p<0.001$) compared with AKH-injected individuals. There was no significant alteration in GSH levels in this case compared with controls.

We found some interesting results when we tested the total antioxidant activity of cell free plasma: PQ treatment alone enhanced the antioxidant capacity of hemolymph compared with controls (ca. 1.7 fold, $p<0.001$) or AKH-injected groups (ca. 1.14 fold, $p<0.01$) (Fig. 7). AKH treatment was effective in enhancing antioxidant capacity (ca. 1.5 fold, $p<0.001$) compared with controls. Co-injection of AKH with PQ resulted in a significant increase in antioxidant capacity of hemolymph compared with all other treatments (PQ ca.1.4 fold - $p<0.001$, AKH ca. 1.5 fold - $p<0.001$, and controls ca. 2.3 fold - $p<0.001$). There were no significant differences in antioxidant capacity of Ringer saline treated controls compared with intact (non-injected) insects (data not shown).

DISCUSSION

A stressor may be defined as a stimulus that disrupts homeostasis in an organism. Oxidative stress represents this type of stressor, whereby the production of free radicals is enhanced in an organism and the antioxidant defense response is impaired. The adaptive responses that are elicited in response to such stressor include physiological processes that are essential to re-establish the homeostatic balance. The roles of neuropeptides and other hormones in this regard are of paramount importance and we have scant information on such aspects. In this work two representatives of two different insect hormone families were chosen – adipokinetic hormones and ecdysteroides – in order to find out possible antioxidative effects of Pyrap-AKH and 20-hydroxy ecdysone (20E) in *Pyrrhocoris apterus* exposed to oxidative stress conditions induced by Paraquat injection.

This study demonstrates that lipid peroxidation in CNS and the carbonyl content in CNS and hemolymph are enhanced, and the content of GSH and the activity of γ-GTP are reduced in the brain of *P. apterus* injected with PQ. The radicals promote lipid peroxidation and carbonyl formation, convert GSH to its oxidized form, and probably damage γ-GTP by its carbonylation or in consequence to the changes caused in the plasma membrane.

The antioxidant properties of 20E

Antioxidant activity has been demonstrated in the vertebrate hormone estrogen and related compounds with the phenolic A-ring (Sugioka et al., 1987; Subbiah et al., 1993; McHugh et al., 1998; Liehr and Roy, 1998). Jellinck and Bradlow (1990) showed that estrogens inhibit oxidative cascades by donating hydrogen radicals on the A-ring. 20E does not harbor any phenolic structure but the widespread occurrence of enzymes for the reversible interconversion of the 3-oxy and 3-dehydroxy compounds with the participation of NAD(P)H (reviewed by Rees, 1995) make the A-ring of 20E and other 2,3-dihydroxy ecdysteroids possible candidate for radical scavenging. Cai et al. (2002) proposed, however, that 20E and three other ecdysteroids tested react with radicals by abstracting hydrogen from carbon 9 at the junction of the B and C rings. They show that the dissociation energy of the C_9-H bond is lower than that of the O-H bond in the efficient antioxidant α-tocopherol.

Lower lipid peroxidation and protein carbonylation following 20E injection are probably direct consequences of a decrease of the oxidative radicals, whereas effects on the glutathione redox shuttle and on the γ-GTP activity represent complex responses. Glutathione is an

important antioxidant that acts by several mechanisms, including scavenging of the free radicals (Meister and Anderson, 1983), but the capacity of this system obviously cannot cope with the burst of oxidative radicals following PQ injection. We assume that considerable recovery of GSH following 20E injection is only partially due to the abrogation of oxidative radicals. The concentration of intracellular GSH depends on the availability of substrates for its synthesis and on the rate of its depletion into the extracellular space (there is only a small or no uptake into the cells) where it is metabolized by the membrane-bound γ-GTP. The enzyme removes the γ-glutamyl group and thereby renders the remaining cysteinylglycine susceptible to cleavage by a membrane dipeptidase. The released cysteine can be transported into the cell and used as a substrate for the GSH synthesis. This γ-glutamyl cycle is thought to sustain the cellular GSH concentration (Karp et al., 2001). The ameliorating 20E effect on the γ-GTP activity, which may be based on the stabilization of its anchoring in the plasma membrane (reduced lipid peroxidation) or on its protection against a direct oxidative damage, contributes to the GSH recovery. Different 20E efficiencies in our assays probably reflect diverse reactivity with the array of oxidative radicals generated by PQ. Cai et al. (2002) demonstrated inactivity with the superoxide.

Females were more resistant than males to all manifestations of the stress and were also more prone to recover after the 20E injection. Higher resistance can be due to (1) larger body size; (2) larger stores of reserve lipids that can absorb radicals by the peroxidation process; (3) presence of antioxidant yolk components such as carotenoids; (4) differences in the titer of endogenous ecdysteroids (Socha, 1993). The last possibility is particularly intriguing because ecdysteroids are known to accumulate in the eggs (Hagedorn, 1985). Analysis of ecdysteroids in *P. apterus* identified makisterone A as the major hormone and disclosed that it was present in low amounts in the body of adults (Zachardová et al., 1989). In contrast to 20E, however, an injection of 1 pmol makisterone A did not exert antioxidative response (preliminary unpublished observation). It should also be noted that 1 pmol 20E we injected provided about 15 ng of the hormone per g body weight which is close to the ecdysteroid baseline that seems to be maintained in the body at all times (Sehnal, 1989). Assuming that the injected 20E was evenly dispersed in the body, it may reach maximally a 1 μM concentration, while concentrations effective in the *in vitro* assays were 20 μM and higher (Cai et al., 2002). It is therefore not excluded that the injected 20E functioned as a trigger to activate an innate antioxidative system.

The antioxidant properties of AKH

In an earlier study on *L. decemlineata* it was demonstrated that AKH titer is increased in CNS and hemolymph as a result of PQ treatment which evokes conditions of oxidative stress (Kodrík et al., 2007). However, in the present investigation no significant change in AKH titer in the CNS was evidenced but a significant elevation of AKH levels in the hemolymph of *P. apterus* male was observed. The reasons for such a differential response could be many, but essentially it mimics a similar situation as observed in case of insecticide treatment (Kodrík and Socha, 2005) that increased the AKH level only in the hemolymph and not in the CNS. It seems that present *Pyrrhocoris* data support a suggestion that the coupling between release and biosynthesis of the AKHs in the adipokinetic cells of the CC is very loose or does not even exist (Diederen et al., 2002).

The importance of insect hormones including the members of the AKH family in stress situations was first pointed out by Ivanovic and Jankovic-Hladni (1991). The recent investigations on insecticide treatment, saline (KCl) treatment, photophase interruption and feeding on genetically modified crops (Candy, 2002; Kodrík and Socha, 2005; Kodrík et al., 2005; 2007) all support idea that AKHs in insects behave as typical stress-responsive hormones. In a series of investigations on the interaction between immune response and AKH on *Locusta migratoria*, it was found that exogenous AKH potentiates the phenoloxidase activity and enhances the nodulation response to injected laminarin and bacterial lipopolysaccharide (Goldsworthy et al., 2002; 2003a, b; 2005). In each of these cases, however, the mechanism of AKH potentiated immune response is far from clear. Hence, our next question was whether enhanced AKH titer in hemolymph upon oxidative stress results in an enhanced antioxidant defense response. To address protein carbonyls were chosen as a reliable biomarker of oxidative stress. Carbonyls are formed from the amino groups in the side chains of certain amino acids that are exposed to reactive oxygen species (Chevion et al., 2000). PQ, as mentioned in the introduction, is a bipyridilium herbicide, easily reduced by a single electron to a stable but dioxygen-sensitive monocation radical ($PQ^{\bullet+}$). The reaction between the PQ radical and dioxygen (O_2) generates the true toxic species, the superoxide radical (O_2^-) and subsequently hydrogen peroxide (H_2O_2). Hydroxyl radicals ($OH^{\bullet}$) may also be generated as a consequence of secondary interactions between O_2^- and H_2O_2 (Hassan, 1984). Hence, one would expect more carbonylation upon PQ treatment. Our results confirmed the presumption: PQ treatment did result in significantly enhanced carbonyl contents in hemolymph, but interestingly co-injection of AKH with PQ decreased their levels to those found in control groups. Also surprising was that AKH injection alone did not change the carbonyl contents to

those below control values. This indicates that possibly a stressor action is needed for AKH to potentiate the response as in case of phenoloxidase activity (Goldsworthy et al., 2002) or lipid stores mobilization rate after injection or topical application of external AKH (Kodrík et al., 2002a). In the latter case it seems that the adipokinetic response is enhanced and or to some extent modified by the stress caused by the injection.

A lowering of the carbonyl contents suggests an antioxidant action by AKH to counter oxidative stress in insects. The mechanism of this antioxidant action is still unclear. As in case of *L. decemlineata* (Kodrík et al., 2007), AKH is also able to enhance efflux of reduced GSH into the plasma of *P. apterus*. In case of glucagon (a pancreatic vertebrate hormone) a similar mechanism has been proposed whereby glucagon down-regulates the synthesis of GSH in the liver while promoting its efflux into the blood plasma (Lu et al., 1990, 1991). However, it is quite possible that GSH alone (induced by AKH) could not be the one to confer enhanced antioxidant capacity to the hemolymph. This was cross-checked, using the total antioxidant activity assay in cell free plasma against Trolox (an analog of vitamin E) standards. This procedure relies on antioxidant activity of low molecular weight compounds. PQ injection alone potentiated an antioxidant response which was significantly enhanced upon co-injection of PQ with AKH. However, AKH injection alone was not capable of inducing antioxidant activity to the levels produced by PQ alone or by co-injection with PQ, though it was more enhanced compared to control groups. Hence it is assumed that there must be some other additional mechanism (other than GSH efflux alone) by which AKH acts to enhance antioxidant response.

Based on these results it is suggested that AKH-potentiated antioxidant response includes a direct or indirect increase of the GSH efflux from the tissues as well as an increased antioxidant capacity of cell free hemolymph plasma by as yet unknown mechanisms. This conclusion is supported by GSH measurements, that in accordance with enhanced carbonyl levels (indicative of oxidative stress), shows a significant decline after PQ treatment, and significant elevation after the AKH/paraquat co-application. Alternatively, AKHs are known to exert their effects through conversion of high density lipoproteins (HDLs) to low density lipoproteins (LDLs), and the latter are more susceptible to peroxidation by free radicals. Thus, increased AKH levels could also result in more LDLs which would act as a sacrificial antioxidant (Miller and Paganga, 1998). This would indicate a more direct role for involvement of AKH in antioxidant response and probably also explain the elevated antioxidant capacity of hemolymph of AKH/PQ treated insects.

GENERAL CONCLUSIONS

The main objectives of this work was to investigate possible antioxidant effects of two insect hormones of two distinctively different hormone families, the adipokinetic hormone (AKH) and 20-hydroxyecdysone (20-HE), in the red firebug *Pyrrhocoris apterus*. The response of these hormones was examined after the Paraquat injection, which evoked in *P. apterus* massive oxidative stress conditions because of well-known toxic activity of this herbicide demonstrated in various plant and animal tissues. The results presented in this thesis confirmed an antioxidant effect of Pyrap-AKH and 20-hydroxyecdysone though the particular mechanism of this protection and how exactly it works is still hidden somewhere in complex enzymatic and hormonal system and awaits further elucidation.

Males proved more susceptible to oxidative stress than females. Particularly in brain membrane fraction, TBARS and protein carbonyls which were both assayed as main oxidative stress biomarkers after PQ application were higher in males than in females demonstrating that females cope with toxic effect of ROS better than males. These sex-related differences were also obvious when oxidative stress was mitigated with 20E: TBARS and protein carbonyl contents were lower and GSH and γ-GTP activity were higher in females indicating better utilization of 20E in females than in males when "fighting" against oxidative stress. 20E also seems to protect the whole organism against the damaging effects of paraquat, which was demonstrated by monitoring the hemolymph protein profile and the female reproduction potential. The 13 kDa protein was absent in hemolymph of insects injected with PQ and present in those supplied with 20E, either alone or in combination with PQ whereas amount of the protein was distinctly higher in females than in males. However, mechanism of this action is unknown.

Experiments with AKH were executed on adult males only since preliminary experiments revealed that males are more responsive to oxidative stress than females. The results demonstrated significantly altered AKH content (even though just in fmol concentrations) in hemolymph upon oxidative stress created as a result of PQ injection. However, in the brains no differences were observed under the same stress conditions.

For assessment of the antioxidant response in hemolymph, two specific parameters were used: the reduced GSH content and the total antioxidant activity of cell free hemolymph. The results lead to the conclusion that AKH potentiates an antioxidant response in the insect body, particularly the hemolymph. AKH injection alone and also AKH+PQ caused considerable

changes in GSH contend compared to PQ injection showing that AKH regulates the GSH pool and so implying an important role of AKH in antioxidant response. Also, total antioxidant activity of hemolymph was significantly increased after AKH alone and AKH+PQ injection compared to PQ injection only. The results are novel and interesting and pave the way for future studies on hormonal regulation of oxidative stress in insects as well as in higher animals.

REFERENCES

Beckman, K. B. and B. N. Ames. Oxidants and aging. In Sen, C. K., L. Packer, and O. Hanninen, eds. Handbook of Oxidants and Antioxidants in Exercise. Amsterdam, Elsevier. 755-796, 2000.

Bielski, B. H. J. and A. O. Allen. Mechanism of the disproportionation of superoxide radicals. *J Phys Chem* 81: 1048-1050, 1977.

Buettner, G. R. The pecking order of free radicals and antioxidants: lipid peroxidation, alpha-tocopherol, and ascorbate. *Arch Biochem Biophys* 300: 535-543, 1993.

Bus JS, Aust SD, Gibson JE. Superoxide and singlet oxygen-catalyzed lipid peroxidation as a possible mechanism of paraquat (methyl viologen toxicity*). Biochem Biophys Res Commun* 58: 749-755, 1974.

Bus JS, Cagen, SZ, Olgaard M, Gibson JE. A mechanism of paraquat toxicity in mice and rabbits. *Toxicol Appl Pharmacol* 35: 501-513, 1976.

Cai Y-J, Dai J-Q, Fang J-G, Ma L-P, Hou L-F, Yang L, Liu Z-L. Antioxidative and free radical scavenging effects of ecdysteroids from *Serratula strangulata*. Can J Physiol. Pharmacol 80: 1187-1194, 2002.

Candy, D.J. Adipokinetic hormones concentrations in the haemolymph of *Schistocerca gregaria*, measured by radioimmunoassay. *Insect Biochem. Mol. Biol.* 32, 1361-1367, 2002.

Chance, B., H. Sies, and A. Boveris. Hydroperoxide metabolism in mammalian organs. *Physiol Rev* 59: 527-605, 1979.

Chevion, M., Berenhstein, E., Stadtman, E.R. Human studies related to protein oxidation: protein carbonyl content as a marker of damage. *Free Rad. Res.* 33 (Suppl.), 99-108, 2000

Diederen, J.H.B., Oudejans, R.C.H.M., Harthoorn, L.F., Van der Horst, D.J. Cell biology of the adipokinetic hormone-producing neurosecretory cells in the locust corpus cardiacum. *Microsc. Res. Techniq*., 56, 227-236, 2002.

Fee, J. A. and J. S. Valentine. Chemical and physical properties of superoxide. In Michelson, A. M., J. M. McCord, and I. Fridovich, eds. Superoxide and Superoxide Dismutases. New York, *Academic Press.* 19-60, 1977.

Fridovich, I. and B. Freeman. Antioxidant defense in the lung. *Annu Rev Physiol* 48: 693-702, 1986.

Frimer, A. A. and I. Rosenthal. Chemical reactions of superoxide anion radicals in aprotic solvents. *Photochem Photobiol* 28: 711-719, 1978.

Gäde G. Regulation of intermediary metabolism and water balance of insects by neuropeptides. *Annu. Rev. Entomol*. 49, 93-113, 2004.

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

Gäde, G., Hoffmann, K.H., Spring, J.H. Hormonal regulation in insects: facts, gaps and future directions. *Physiol. Rev*. 77, 963-1032, 1997.

Gerschman, R., Gilbert, DI., Nye, SW., Dwyer, P., Fenn, WO. Oxygen poisoning and X-irradiation: A mechanism in common. *Science.* **119:** 623-626, 1954.

Goldsworthy, G.J., Chandrakant, S., Opoku-Ware, K. Adipokinetic hormone enhances nodule formation and phenoloxidase activation in adult locusts injected with bacterial lipopolysaccharide. *J. Insect Physiol*. 49, 795-803, 2003a.

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

Goldsworthy, G.J., Opoku-Ware, K., Mullen, L.M. Adipokinetic hormone and the immune responses of locusts to infection. *Ann. N.Y. Acad. Sci*. 1040, 106-113, 2005.

Goldsworthy, G.J., Mullen, L., Opoku-Ware, K., Chandrakant, S. Interactions between the endocrine and immune systems in locusts. *Physiol. Entomol*. 28, 54-61, 2003b.

Griffith OW. Determination of glutathione and glutathione disulfide using glutathione reductase and 2-vinylpyridine. *Anal Biochem* 106: 207-212, 1980.

Hagedorn HH. The role of ecdysteroids in reproduction. In: Kerkut GA, Gilbert LI, editors. Comprehensive Insect Physiology, Biochemistry and Pharmacology. Oxford: *Pergamon Press*, vol 8, p 205-262, 1985.

Halliwell, B., Gutteridge, J.M.C. Free Radicals in Biology and Medicine, *Clarendon Press*, Oxford, 1989.

Hansen, K.K., Hauser, F., Cazzamali, G., Williamson, M. & Grimmelikhuijzen C.J.P. Cloning and characterization of the adipokinetic hormone receptor from the cockroach *Periplaneta americana. Biochemical and Biophysical Research Communication,* **343**, 638-643, 2006.

Hassan, H.M. Exacerbation of superoxide radical formation by paraquat. *Methods Enzymol*. 105, 523-532, 1984.

Hassan, H. M. Cytotoxicity of oxyradicals and the evolution of superoxide dismutase. In Clerch, L. B. and D. J. Massaro, eds. Oxygen, Gene Expression, and Cellular Function. New York, Marcel Dekker, Inc. 27-47, 1997.

Hassan HM, Fridovich I. Intracellular production of superoxide radical and hydrogen peroxide by redox active compounds. *Arch Biochem Biophys* 196: 385-395, 1979.

Ikeda Y. γ-Glutamyl transpeptidase. In : Taniguchi N, Gutteridge JMC. editors. Experimental protocols for reactive oxygen and nitrogen species. Oxford Univ Press, London. p 86-87, 2000.

Imlay, J.A. and Linn, S. DNA damage and oxygen radical toxicity. *Science* 240:1302-1309, 1986.

Ivanovic, J., Jankovic-Hladni, M. Hormones and metabolism in insect stress. *CRC Press*, Boca Raton, 1991.

Jellinck PH, Bradlow HL. Peroxidase-catalyzed displacement of tritium from regiospecifically labeled estradiol and 2-hydroxyestradiol. *J Steroid Biochem* 35: 705-710, 1990.

Karp DR, Shimooku K, Lipsky PE. Expression of γ-glutamyl transpeptidase protects Ramos B cells from oxidation-induced cell death. *J Biol Chem* 276: 3798-3804, 2001.

Kaufmann, C. & Brown, M.R. Adipokinetic hormone in the African malaria mosquito, *Anopheles gambiae*: Identification and expression of genes for two peptides and a putative receptor. *Insect Biochemistry and Molecular Biology*, **36**, 466-481, 2006.

Kelner MJ, Bagnell R. Glutathione –dependent enzymes alone can produce paraquat resistance. *Free Rad Biol Med* 9: 149-153, 1990.

Klein JA, Ackerman SL. Oxidative stress, cell cycle and neurodegeneration. *J Clin Invest* 111: 785-793, 2003.

Kodrík, D., Krishnan, N., Habuštová, O. Is the titre of adipokinetic peptides in *Leptinotarsa decemlineata* fed on genetically modified potatoes increased by oxidative stress? Peptides doi:10.1016/j.peptides.2007.01.017 (in press), 2007.

Kodrík, D., Socha, R. The effect of insecticide on adipokinetic hormone titre in the insect body. *Pest. Manag. Sci.* 61, 1077-1082, 2005.

Kodrík, D., Socha, R., Syrová, Z., 2003. Developmental and diel changes of adipokinetic hormone in CNS and haemolymph of the flightless wing-polymorphic bug, *Pyrrhocoris apterus*. J. Insect Physiol. 49, 53-61.

Kodrík, D., Socha, R., Syrová, Z., Zemek, R. The effect of constant darkness on the content of adipokinetic hormone, adipokinetic response and walking activity in macropterous females of *Pyrrhocoris apterus* (L.). *Physiol. Entomol*. 30, 248-255, 2005.

Kodrík, D., Socha, R., Šimek, P., Zemek, R., Goldsworthy, G.J. A new member of the AKH/RPCH family that stimulates locomotory activity in the firebug, *Pyrrhocoris apterus* (Heteroptera). *Insect Biochem. Mol. Biol.* 30, 489-498, 2000.

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

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

Krishnan N., Večeřa J., Kodrík D., Sehnal F. 20-hydroxyecdysone prevents oxidative stress damage in adult *Pyrrhocoris apterus*. *Arch. Insect Biochem. Physiol*. (in press), 2007b.

Kukreja, R. C. and M. L. Hess. Oxygen radicals and myocardial injury. In Kukreja, R. C. and M. L. Hess, eds. Free Radicals, Cardiovascular Dysfunction and Protection Strategies. Austin, Texas, R.G.Landes Company. 28-39, 1994.

Lacort M, Leal AM, Liza M, Martin C, Martinez R, Ruiz-Larrea MB. Protective effects of estrogens and catecholestrogens against peroxidative membrane damage in vitro. *Lipids* 30: 141-146, 1995.

Levine, R.L., Garland, D., Oliver, C.N., Amici, A., Climent, I., Lenz, A.G. Determination of carbonyl content in oxidatively modified proteins. Methods Enzymol. 186, 464-478, 1990.

Liehr JG, Roy D. Pro-oxidant and antioxidant effects of estrogens In: Armstrong D editor, Methods in Molecular Biology, Vol.108: Free radical and antioxidant protocols. *Humana Press Inc*., Totowa, NJ. p 425- 435, 1998.

Lu, S.C., Garcia-Ruiz, C., Kuhlenkamp, J., Ookhtens, M., Salas-Prato, M., Kaplowitz, N. Hormonal regulation of glutathione efflux. *J. Biol. Chem.* 265, 16088-16095, 1990.

Lu, S.C., Kuhlenkamp, J., Garcia-Ruiz, C., Kaplowitz, N. Hormone-mediated down-regulation of hepatic glutathione synthesis in the rat. *J. Clin. Invest*. 88, 260-269, 1991.

Lynch, R. E. and I. Fridovich. Permeation of the erythrocyte stroma by superoxide radical. *J Biol Chem* 253: 4697-4699, 1978.

McCord, JM., Fridovich, I. Superoxid dismutase. *J. Biol. Chem.* **244:** 6049-6055, 1969.

McHugh NA, Merrill GF, Powell SR. Estrogen diminishes postischemic hydroxyl radical formation. Am *J Physiol Heart Circ Physiol* 274: H1950-H1954, 1998.

Meister, A. Glutathione metabolism and its selective modification. *J. Biol. Chem.* 263:17205-17208, 1988.

Meister A, Anderson ME. Glutathione. *Annu Rev Biochem* 52: 711-760, 1983.

Milde, J.J., Ziegler, R. & Wallstein, M. Adipokinetic hormone stimulates neurons in the insect central nervous system. *The Journal of Experimental Biology*, **198**, 1307-1311, 1995.

Miller, N.J., Paganga, G. Antioxidant activity of low-density lipoprotein. In: Armstrong D (Ed.) Methods in Mol. Biol., Vol. 108, Free radical and antioxidant protocols. *Humana Press*, pp. 325-335, 1998.

Mullen, L., Goldsworthy, G. Changes in lipophorins are related to the activation of phenoloxidase in the haemolymph of *Locusta migratoria* in response to injection of immunogens. *Insect Biochem. Mol. Biol*. 33, 661-670, 2003.

Nishikimi, M. and K. Yagi. Oxidations of ascorbic acid and _-tocopherol by superoxide. In Hayyaishi, O. and K. Asada, eds. Biochemical and Medical Aspects of Active Oxygen. Tokyo, *University of Tokyo Press*. 79-87, 1977.

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

Patel, K. B. Semiquinone free radicals and oxygen: pulse radiolysis study of one electron transfer equilibriums. *J Chem Soc Faraday Trans* 69: 814-825, 1973.

Rees HH. Ecdysteroid biosynthesis and inactivation in relation to function. *Eur J Entomol* 92: 9-39, 1995.

Rennenberg, H.Glutathione metabolism and possible biological roles in higher plants. *Phytochem.* 21:2771-2781, 1982.

Rice-Evans, C., Miller, N.J. Total antioxidant status in plasma and body fluids. Methods Enzymol. 234, 279-293, 1994.

Richter, C. Biophysical consequences of lipid peroxidation in lipid membranes. *Chem Phys Lipids* 44: 175-189, 1987.

Samaranayaka, M. Insecticide-induced release of hyperglycaemic and adipokinetic hormones of *Schistocerca gregaria*. *Gen. Comp. Endocrinol*. 24, 424-436, 1974.

Sehnal F. Hormonal role of ecdysteroids in insect larvae and during metamorphosis. In: Koolman J. editor, Ecdysone. Georg Thieme-Verlag, Stuttgart, p. 271-278, 1989.

Shu H, Talcott RE, Rice SA, Wei ET. Lipid peroxidation and paraquat toxicity. *Biochem Pharm* 28: 327-331, 1979.

Socha, R. *Pyrrhocoris apterus* (Heteroptera) – an experimental model species: a review. *Eur. J. Entomol.* 1, 241-286, 1993.

Staubli, F., Jorgensen, T.J.D., Cazzamali, G., Williamson, M., Lenz, C., Sondergaard, L., Roepstorff, P. & Grimmelikhuijzem, C.J.P. Molecular identification of the insect adipokinetic hormone receptors. *Proceedings of the National Academy of Science of the United States of America*, **99**, 3446-3451, 2002.

Subbiah MT, Kessel B, Agrawal M, Rajan R, Abplanalp W, Rymaszewski Z. Antioxidant potential of specific estrogens on lipid peroxidation. *J Clin Endcrinol Metab* 77: 1095-1097, 1993.

Sugioka K, Shimosegawa Y, Nakano M. Estrogens as natural antioxidants of membrane phospholipids peroxidation. *FEBS Lett* 210: 37-39, 1987.

Suzuki, K., H. Ohno, S. Oh-ishi, T. Kizaki, T. Ookawara, J. Fujii, Z. Radak, and N. Taniguchi. Superoxide dismutases in exercise and disease. In Sen, C. K., L. Packer, and O. Hanninen, eds. Handbook of Oxidants and Antioxidants in Exercise. Amsterdam, Elsevier. 243-295, 2000.

Taniguchi S, Yanase T, Kobayashi K, Takayanagi R, Haji M, Umeda F, Nawata H. Catechol estrogens are more potent antioxidants than estrogens for the $Cu(^{2+})$-catalyzed oxidation of low or high density lipoprotein: antioxidant effects of steroids on lipoproteins. *Endocrine J* 41: 605-611, 1994.

Trush MA, Mimnaugh EG, Ginsburg E, Gram TE. In vitro stimulation by paraquat of reactive-oxygen mediated lipid peroxidation in rat lung microsomes. *Toxicol Appl Pharmacol* 60: 279-286, 1981.

Uchiyama M, Mihara M. Determination of malondialdehyde precursor in tissues by the thiobarbituric acid test. *Anal Biochem* 86: 271-278, 1978.

Vanden Hoek, T. L., L. B. Becker, Z. Shao, C. Li, and P. T. Schumacker. Reactive oxygen species released from mitochondria during brief hypoxia induce preconditioning in cardiomyocytes. *J Biol Chem* 273: 18092-18098, 1998.

Večeřa, J., Krishnan, N., Alquicer, G., Kodrík, D., Socha, R. Adipokinetic hormone-induced enhancement of antioxidant capacity of *Pyrrhocoris apterus* hemolymph in response to oxidative stress. *Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology* (in press), 2007.

Wallace, S. S. Enzymatic processing of radiation-induced free radical damage in DNA. *Radiat Res* 150: S60-S79, 1998.

Warner, B. B. and J. R. Wispe. Transgenic models for the study of lung injury and repair. In Clerch, L. B. and D. J. Massaro, eds. Oxygen, Gene Expression, and Cellular Function. New York, Marcel Dekker, Inc. 1997, 75-97.

Wicher, D., Walther, C. & Penzlin, H. Neurohormone-D induces ionic current changes in cockroach central neurons. *Journal of Comparative Physiology*, **174A**, 507-515, 1994.

Zachardová D, Sehnal F, Landa V. Makisterone A content and gonadal development in *Pyrrhocoris apterus* reared under long versus short photoperiods. In: Tonner M, Soldan T, Bennettova B. editors, Regulation of Insect Reproduction, *Academia*, Praha, p. 59-71, 1989.

SUPPLEMENT 1.

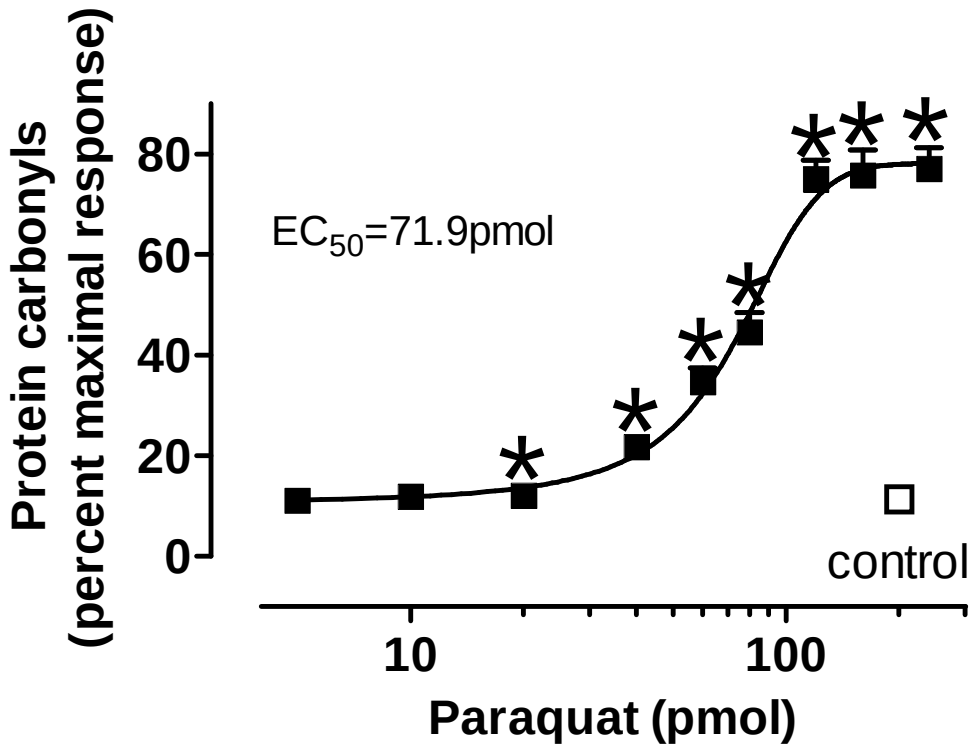

Fig. 1. Effect of increasing doses of paraquat on protein carbonyls level in hemolymph of *P. apterus* adult males expressed as percentage of maximal response. Controls were injected with Ringer saline. Significant differences ($p<0.05$, experimental vs. control) computed using t-test are indicated by asterisks.

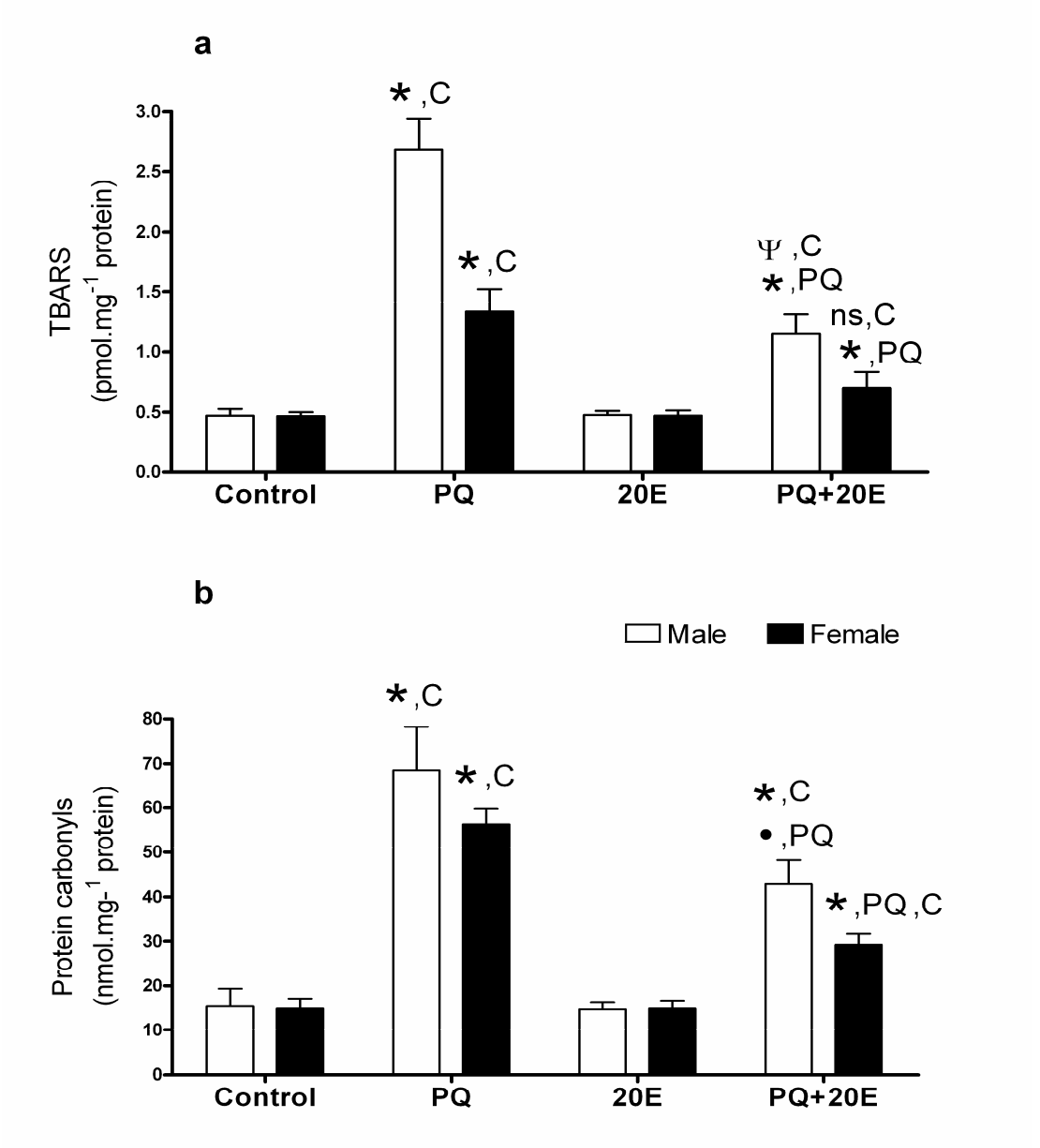

Fig. 2. Thiobarbituric acid reactive substances (TBARS) **(a)** and protein carbonyls **(b)** in the brain microsomal membrane fractions of bugs injected with saline (Control), 38 pmol paraquat (PQ), 1 pmol 20-hydroxyecdysone (20E), and both PQ and 20E. Data are means ± SE; n = 9 for TBARS and 15 for protein carbonyls. Significance of differences between the treatments within a sex are shown above the columns (*$p<0.0001$, $^{\Psi}p<0.001$, •$p<0.01$, ^{ns}not significant). PQ and PQ+20E treatments caused significantly ($p<0.001$ and $p<0.05$, respectively) more TBARS in the male than the female brains. Protein carbonyls were significantly ($p<0.01$) lower in females only after the co-injection of PQ with 20E.

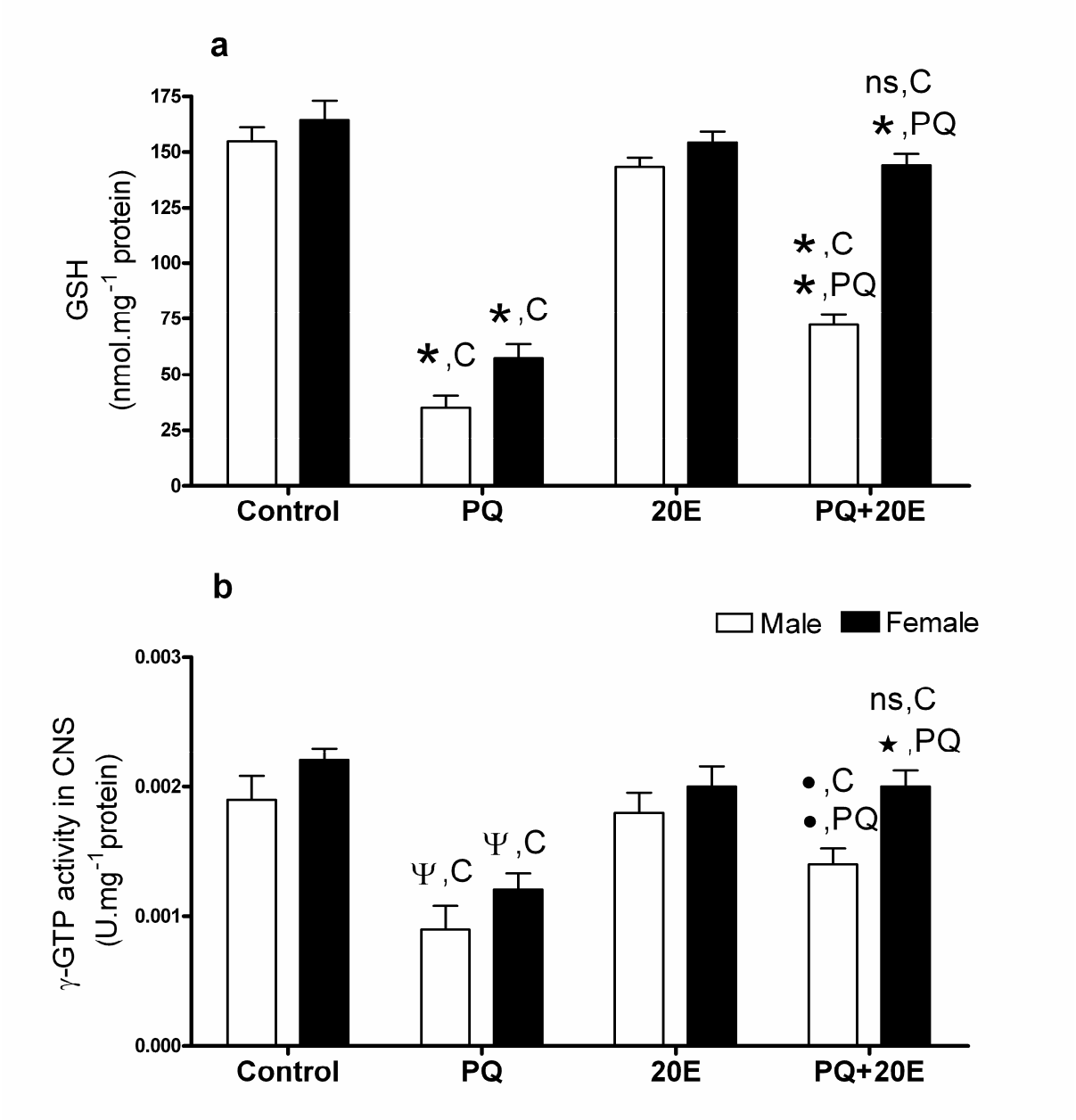

Fig. 3. Reduced glutathione (GSH) **(a)** and activity of γ-glutamyl transpeptidase (γ-GTP) **(b)** in the brain of bugs injected with saline (Control), 38 pmol paraquat (PQ), 1 pmol 20-hydroxyecdysone (20E), and both PQ and 20E. Data are means ± SE, n = 9. Significance of differences between the treatments within a sex are shown above the columns (*$p<0.0001$, $^{\Psi}p<0.005$, •$p<0.03$, $^{\star}p<0.001$, ^{ns}not significant). γ-GTP activity was significantly ($p<0.003$) higher in females than in males after the co-injection of PQ with 20E.

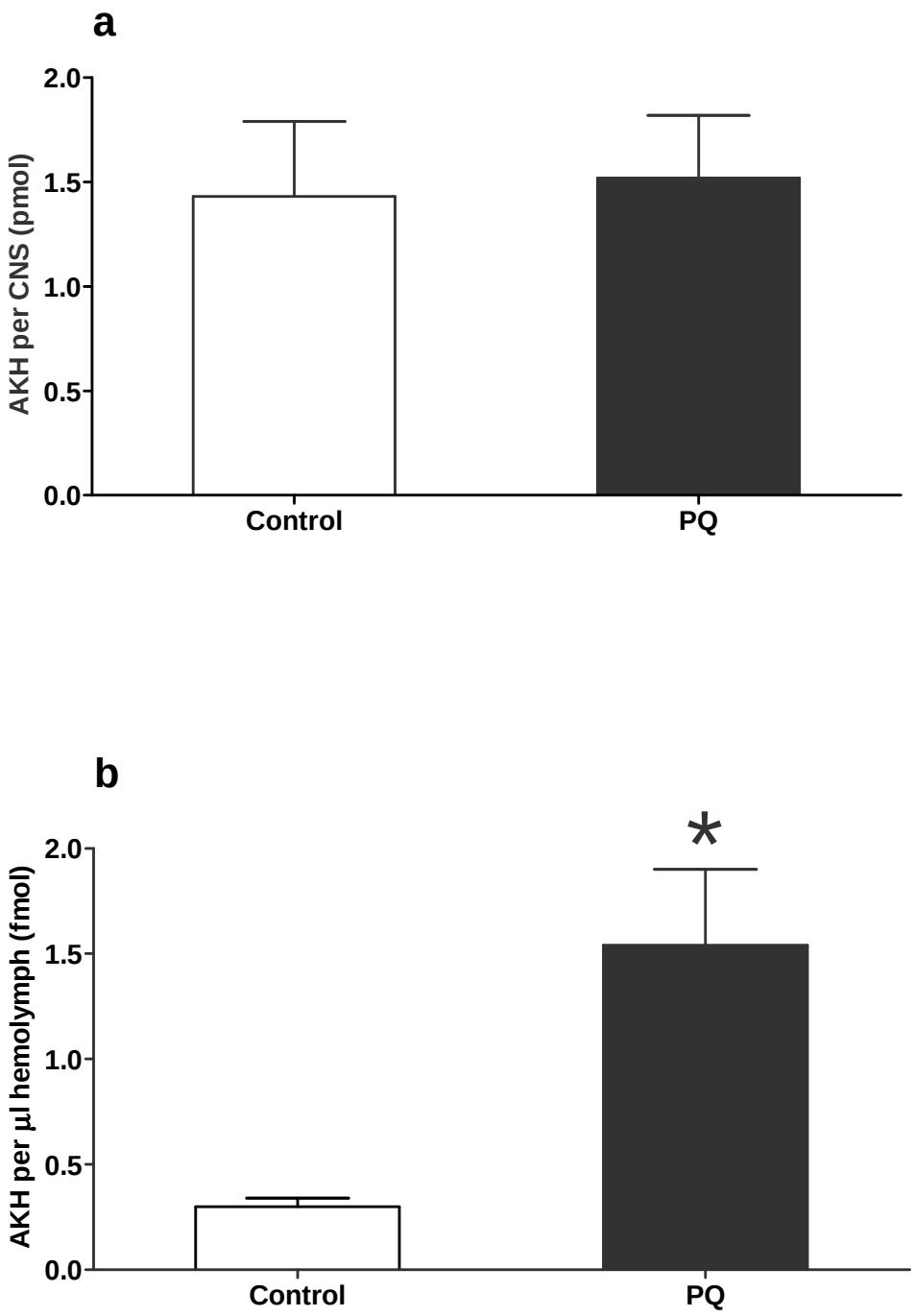

Fig 4. Effect of paraquat (40 pmol) injection on the AKH level in CNS (A) and hemolymph (B) of *P. apterus* adult males. Controls were injected with Ringer saline. Significant difference ($p<0.05$, experimental vs. control) computed using t-test is indicated by an asterisk.

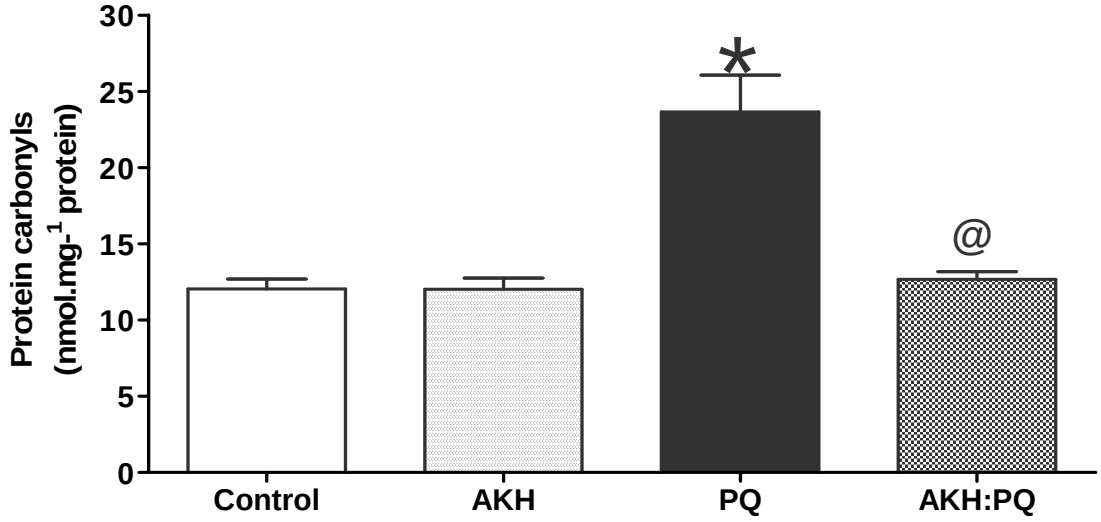

Fig 5. Effect of AKH (Pyrap-AKH, 40 pmol) and paraquat (40 pmol) injection on protein carbonyl content in hemolymph of *P. apterus* adult males. Controls were injected with Ringer saline. Significant differences ($p<0.01$) computed using Tukey's multiple comparison test following one-way ANOVA are indicated as follows: paraquat vs. control by an asterisk, paraquat:AKH vs. paraquat by @.

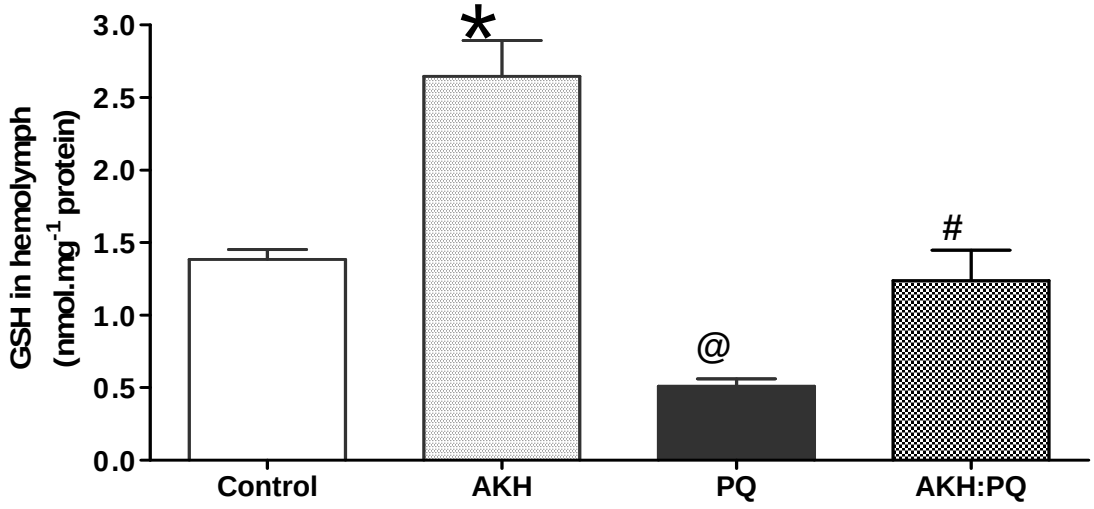

Fig. 6. Effect of AKH (Pyrap-AKH, 40 pmol) and paraquat (40 pmol) injection on glutathione content in hemolymph of *P. apterus* adult males. Controls were injected with Ringer saline. Significant differences computed using Tukey's multiple comparison test following one-way ANOVA are indicated as follows: AKH vs. control by an asterisk ($p<0.001$), paraquat vs. control by @ ($p<0.05$) and paraquat:AKH vs. paraquat by # ($p<0.05$).

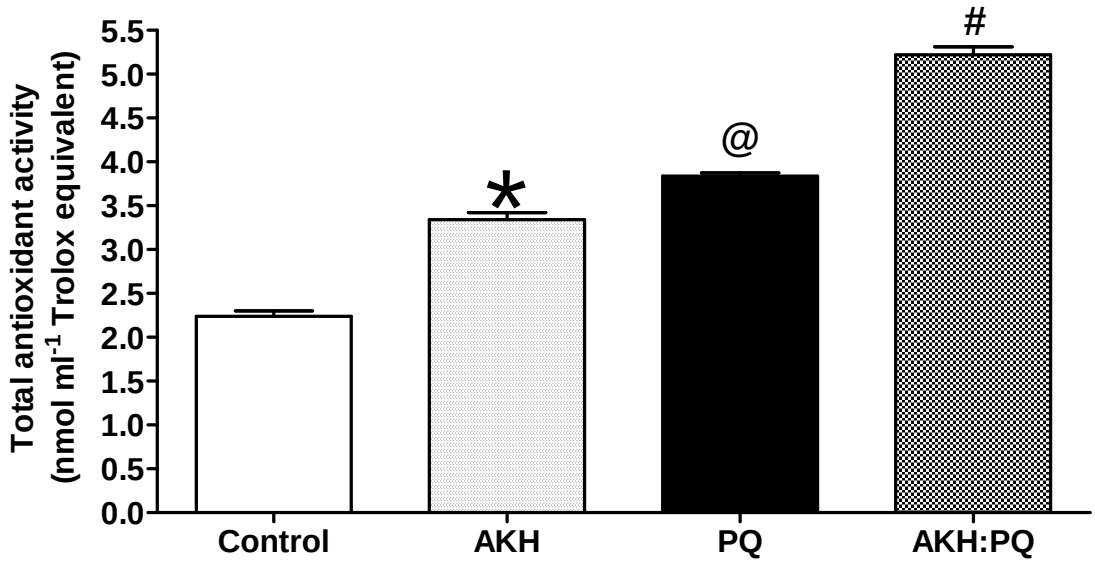

Fig 7. Effect of AKH (Pyrap-AKH, 40 pmol) and paraquat (40 pmol) injection on total antioxidant hemolymph capacity of *P. apterus* adult males. Controls were injected with Ringer saline. Significant differences ($p<0.001$) computed using Tukey's multiple comparison test following one-way ANOVA are indicated as follows: AKH vs. control by an asterisk, paraquat vs. control by @ and paraquat:AKH vs. paraquat by #.

Pictures

Picture 1: Chemical structure of ecdysteroids

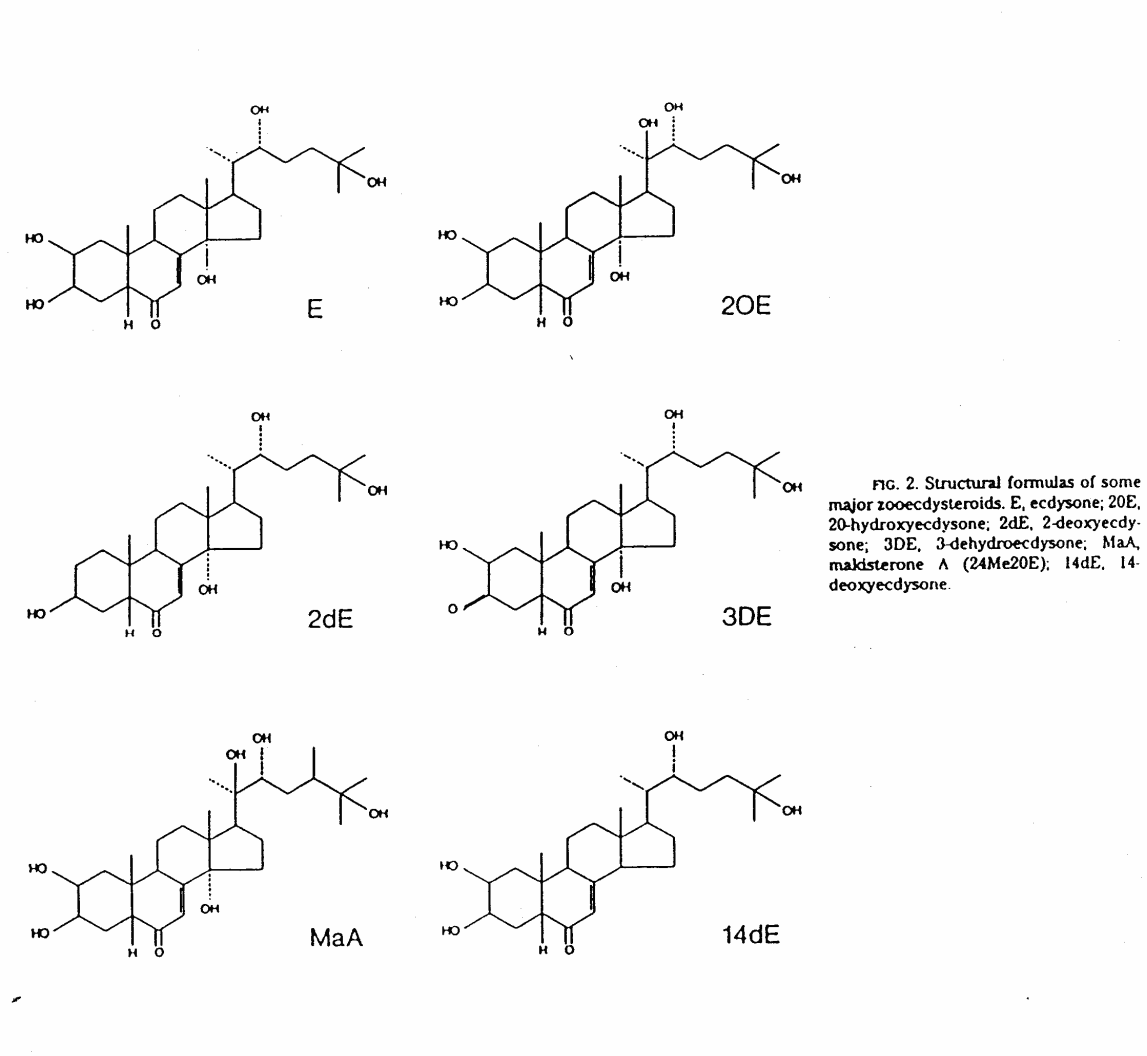

FIG. 2. Structural formulas of some major zooecdysteroids. E, ecdysone; 20E, 20-hydroxyecdysone; 2dE, 2-deoxyecdysone; 3DE, 3-dehydroecdysone; MaA, makisterone A (24Me20E); 14dE, 14-deoxyecdysone.

Picture 2: Ecdysteroids – sites of action in insect body

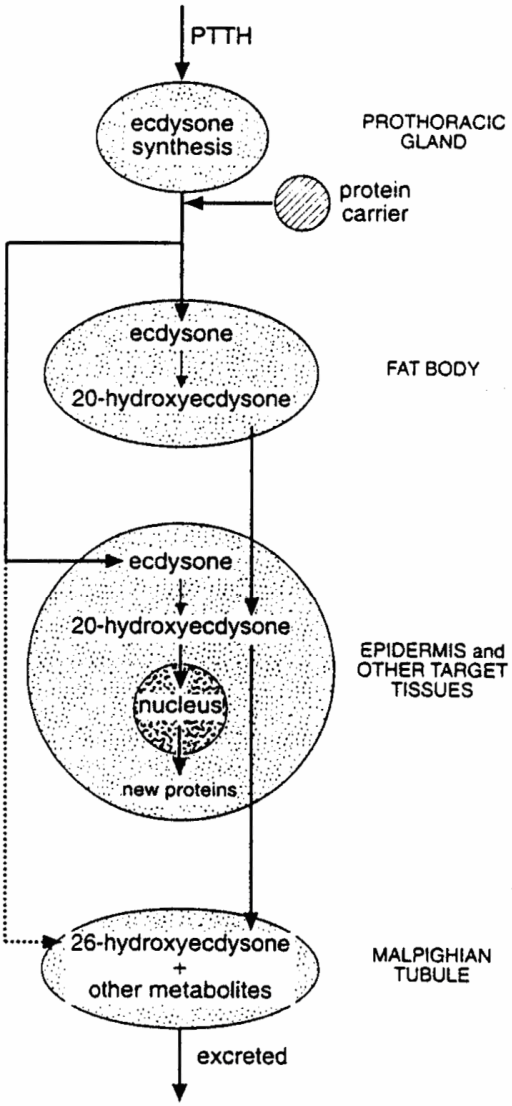

Fig. 21.9. Ecdysteroids. The principal stages in the production, activity and degradation of ecdysteroids.

Picture 3: Role of AKH in energy metabolism

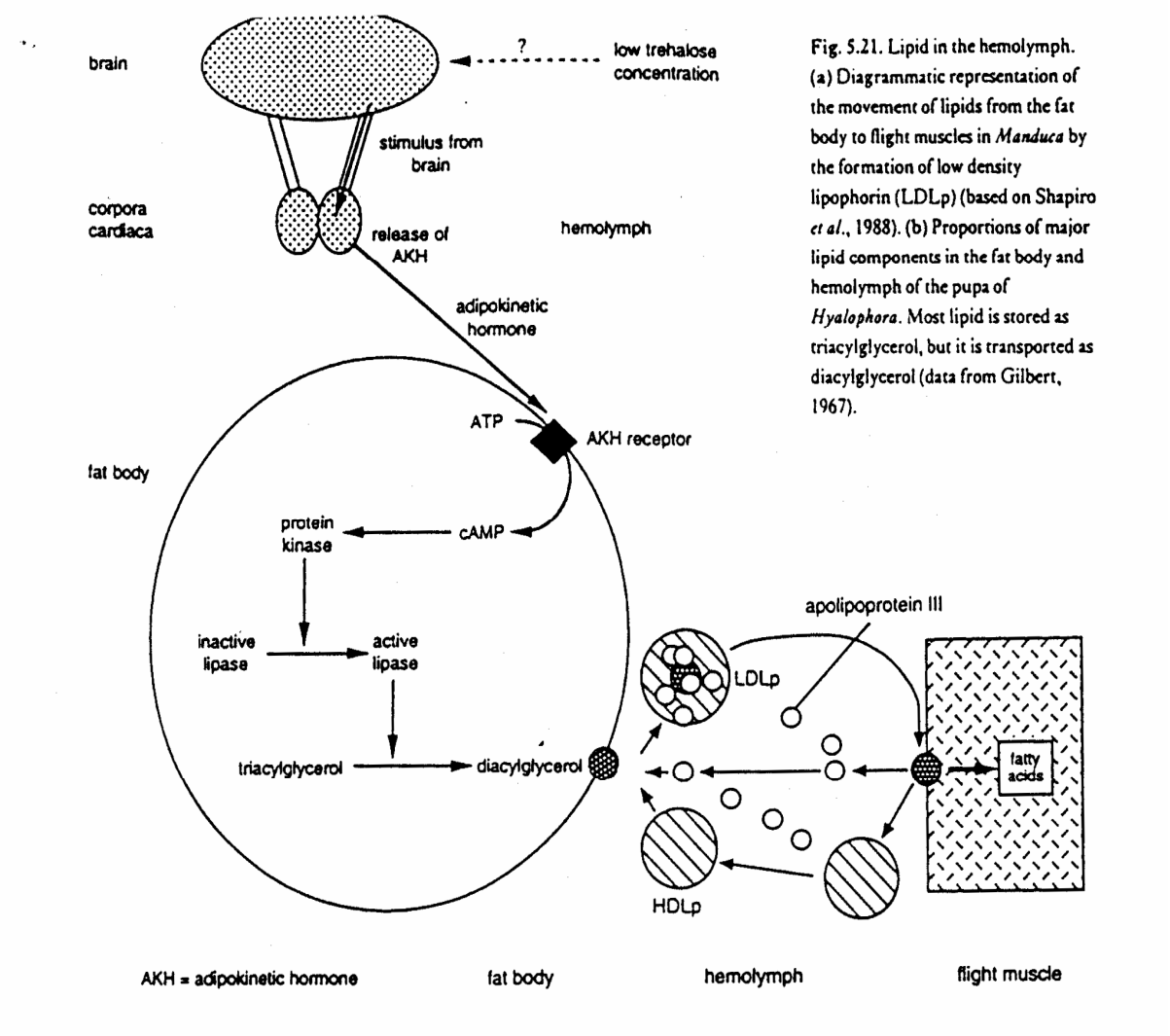

Fig. 5.21. Lipid in the hemolymph. (a) Diagrammatic representation of the movement of lipids from the fat body to flight muscles in *Manduca* by the formation of low density lipophorin (LDLp) (based on Shapiro *et al.*, 1988). (b) Proportions of major lipid components in the fat body and hemolymph of the pupa of *Hyalophora*. Most lipid is stored as triacylglycerol, but it is transported as diacylglycerol (data from Gilbert, 1967).

Picture 4: Paraquat molecule

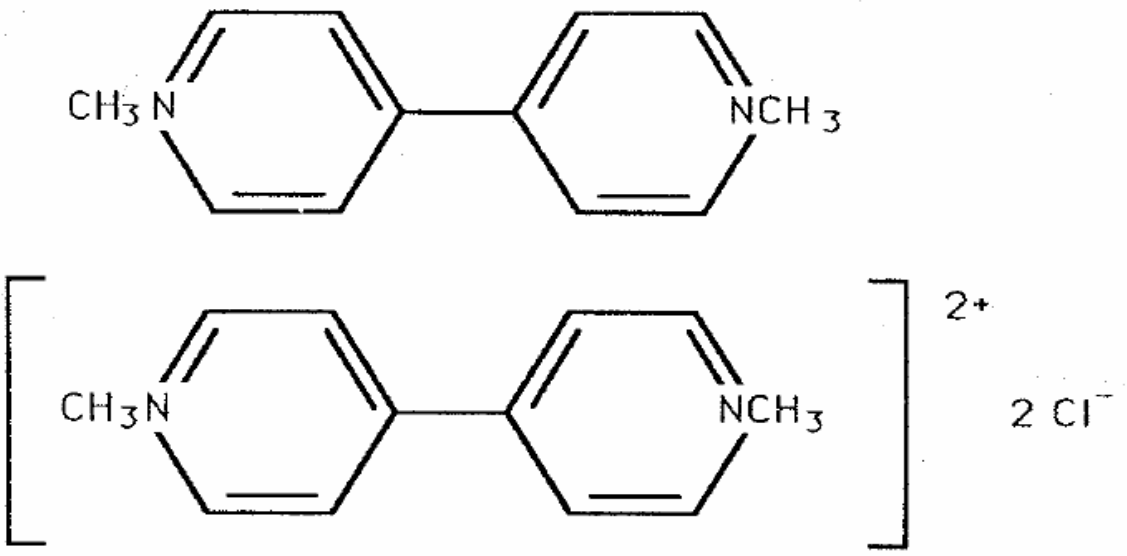

Fig. 1. Structural formula of paraquat cation (*upper*) and of paraquat dichloride salt (*lower*).

SUPPLEMENT 2.

List of publications relating to my thesis

Posters presented at international conferences

Kodrík, D., **Večeřa, J**., Krishnan, N. Oxidative stress modulates the titre of adipokinetic hormone in *Pyrrhocoris apterus* (Heteroptera, Insecta). *23rd Conference of European Comparative Endocrinologists,* The University of Manchester , United Kingdom, 2006.

Krishnan, N., **Večeřa, J**., Kodrík, D., Sehnal, F. 20-hydroxyecdysone inhibits lipid peroxidation and maintains membrane fluidity in the brain of *Pyrrhocoris apterus* subjected to oxidative stress. *16th International Ecdysone Workshop,* Ghent University, Belgium, 2006.

Alquicer, G., Krishnan, N., **Večeřa, J**., Kodrík, D., Socha, R. Does adipokinetic hormone potentiate an antioxidative response to counter oxidative stress in insect? *37th Western Regional Conference on Comparative Endocrinology,* University of Washington, USA, 2007.

Papers "in press" in journals

Krishnan, N., **Večeřa, J**., Kodrík, D., Sehnal, F. 20-hydroxyecdysone prevents oxidative stress damage in adult *Pyrrhocoris apterus. Arch. Insect Biochem. Physiol.*, 2007.

Večeřa, J., Krishnan, N., Alquicer, G., Kodrík, D., Socha, R. Adipokinetic hormone-induced enhancement of antioxidant capacity of *Pyrrhocoris apterus* hemolymph in response to oxidative stress. *Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology* (in press), 2007.