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The role of insect adipokinetic hormones in oxidative stress

Ph.D. Thesis

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Annotation

In this work, the role of adipokinetic hormones (AKHs) in specific pathological conditions known as oxidative stress (OS) was evaluated in insects. A previous suggestion that the AKHs reverse the OS status elicited by the herbicide paraquat was confirmed using other OS elicitor insecticides malathion and endosulfan in the classical model species the firebug *Pyrrhocoris apterus*. Nevertheless, the main part of this work is focused on another insect model a herbivore *Spodoptera littoralis* (Lepidoptera), where the OS was elicited by tannic acid and where the impact of both OS and AKH treatment was evaluated in larval midgut tissue. Several markers of OS including activities of antioxidant enzymes, their gene expression, protein carbonyls and reduced glutathione level were measured. The results confirm the active role of AKH in activation of antioxidative defence reactions and suggest a pathway partially through which the antioxidant effects of AKHs could be realized.

Declaration [in Czech]

Prohlašuji, že svoji disertační práci jsem vypracoval samostatně pouze s použitím pramenů a literatury uvedených v seznamu citované literatury.

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České Budějovice, 15.12.2011 .....

Josef Večeřa

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List of papers and author's contribution

Paper 1

Večeřa, J., Krishnan, N., Mithöfer, A., Vogel, H., Kodrík, D. Adipokinetic hormone-induced antioxidant response in *Spodoptera littoralis. Comp. Biochem. Physiol. C*., in press
Josef Večeřa designed and realized all experiments except for protein carbonyls and mRNA quantification. He is responsible for data assembling, statistical analysis and writting the manuscript.

Paper 2

Velki, M., Kodrík, D., Večeřa, J., Hackenberger, B.K., Socha, R., 2011. Oxidative stress elicited by insecticides: a role for the adipokinetic hormone. *Gen. Comp. Endocrinol.* 172, 77-84.
Josef Večeřa participated in method optimization (sample preparation, assay for catalase activity), quantification of glutathione content and revision of the manuscript.

Paper 3

Alquicer, G., Kodrík, D., Krishnan, N., Večeřa, J., Socha, R., 2009. Activation of insect anti-oxidative mechanisms by mammalian glucagon. *Comp. Biochem. Physiol. B* 152, 226-233.
Josef Večeřa participated in method optimization (quantification of protein carbonyls and glutathione content).

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(in behalf of all above listed co-authors who are not available in Czech Republic)

Contents

Introduction

Embarking on research on aspects involving insect endocrinology and oxidative stress brings with it certain inherent complications because of a relatively low number of published literature or data available on this topic. In my investigations, I have attempted to uncover a role of insect adipokinetic hormones (AKHs) during oxidative stress (OS, see below) conditions elicited by various stressors which are able to induce a massive production of reactive oxygen species (ROS). The AKH, which is a peptide primarily participating in energy metabolism in probably most insect species, was in the last decade found to be involved also in some additional functions that would not necessarily be related to energy metabolism. There are studies demonstrating that AKH level in various insects elevates during stress conditions after application of toxins (1) and insecticides (2-5), or suggesting a certain role of AKH in immune response after the immunogen treatment (6-10). Some of these observations also indicate a feed-back regulation between oxidative stressors and AKH action, and the possible involvement of AKH in antioxidative protective reactions (1-3,11,12). However, the mechanisms of the mode of AKH action (direct or indirect) and their possible differences among the insect species, are far from clear and demand intensive and extensive investigations. In this study, it was thought worth investigating the putative role of AKH in mitigating oxidative stress elicited by oxidative stressors in insect model systems. The question however remains on what are the specific pathways by which AKH initiates possible signaling cascades culminating in the observed antioxidant effects and it is felt that the full answer to this question is not possible through this thesis since the main emphasis given here is on the overt antioxidative actions of AKH in response to OS. In fact, this study encompasses a comprehensive investigation on the overall antioxidative action and potential of AKH to respond to OS, rather than probing into the details of a particular pathway involved in the process, which would be objectives for further research. This was thought to be the proper way to investigate the role of AKH in OS responses in insects. It is felt that the work presented herein would pave the way for a greater understanding of the endocrine system in regulation of OS responses in insects and help in initiating studies on the development of biorational pesticides in the field of entomology and also open new vistas of research on endocrine regulation of response to OS in the field of biomedicine.

1. Reactive oxygen species: formation, function and detoxification

The oxygen molecule is a crucial component of the "cellular breathing" in almost every tissue compartment of aerobic organisms. Under both normal and pathological conditions, oxygen can be relatively easily transformed into highly reactive ROS, such as superoxide radical ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2) or hydroxyl radical ($OH^{\bullet}$). The ROS are generated by several mechanisms in the cell, such as the electron transport chains in membranes and by enzymes producing superoxide anion such as phagocytic and non-phagocytic NADPH oxidases (14) or cytosolic xanthine oxidase (15). A substantial part of ROS (usually over 90%) in living organisms is produced by electron-transport chains - mitochondrial, endoplasmic reticulum, plasma and nuclear membranes, and photosynthetic system (13,16).

Evidences have been accumulated in the last 40 years that ROS can act as both beneficial and deleterious agents depending on concentration in which they are present in the cells but also on the momentary antioxidant potential of target tissue exposed to oxygen radicals. Deleterious effects of ROS are usually consequences of impaired cellular defense mechanisms or uncontrollable burst of ROS within tissues that both lead to pathological conditions called oxidative stress (see section 2) and will be discussed later. Beneficial effects of ROS occur at low/moderate concentrations and involve physiological roles in defense against infectious agents and in the function of a number of cellular signaling systems (Fig. 1). Briefly, low intensity of ROS formation is mainly sensed by the Keap1/Nrf2 system which up-regulates downstream genes encoding antioxidant enzymes in animals (17,18). Intermediate ROS concentrations also increase the activity of antioxidant enzymes, but mainly via the NF-kB and AP-1 pathways (19,20). At both low and intermediate ROS concentrations, the MAP- and other kinases including protein kinase C are also involved in signal sensing and cellular response, leading to enhanced antioxidant potential (21,22). At the molecular level, most of the ROS-regulatory effects are realized through either phosphorylation or reversible modification of specific cysteine residues of target regulatory proteins leading to activation or release of specific transcription factors and expression of target antioxidant genes, respectively.

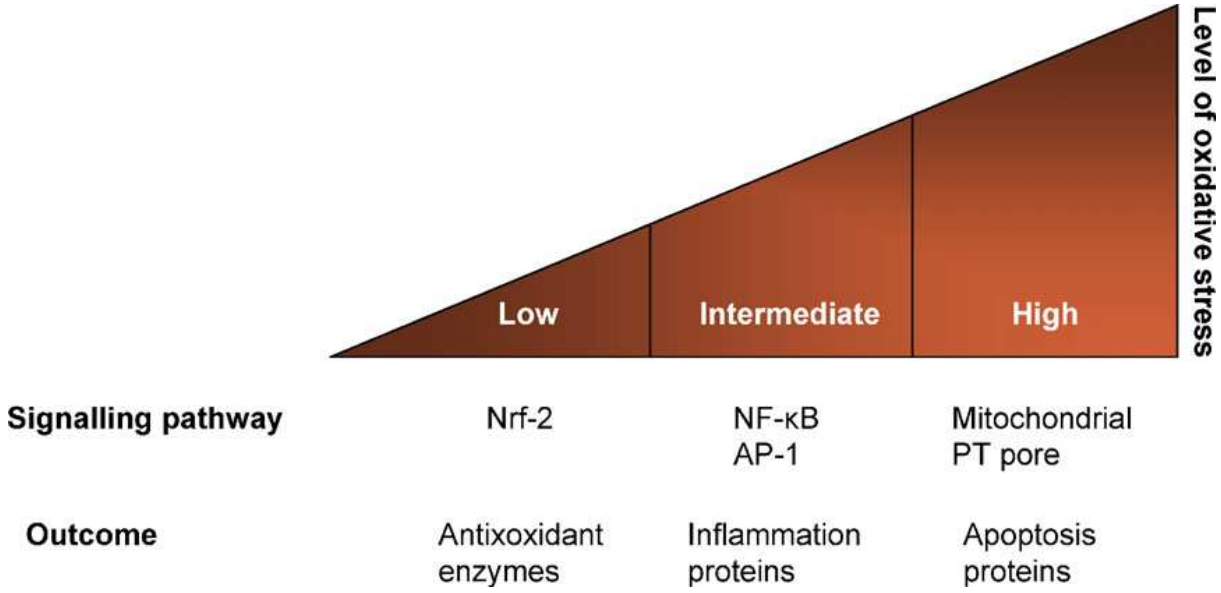

Fig. 1 – Model of OS-initiation treshold. A low ROS formation induces Nrf2, a transcription factor implicated in the transactivation of gene coding for antioxidant enzymes. An intermediate amount of ROS triggers an inflammatory response through the activation of NF-kB and AP-1, and a high amount of oxidative stress induces perturbation of the mitochondrial PT pore and disruption of the electron transfer, thereby resulting in apoptosis or necrosis (21, modified).

1.1 Superoxide radical – *"Initiator"*

Mitochondrial respiration is not the only process which can generate superoxide radical (see section 2.1). Arising either through metabolic processes or following oxygen "activation" by physical irradiation, $O_2^{\bullet-}$ is considered the "primary" ROS, and can further interact with other molecules to generate "secondary" ROS, either directly or prevalently through enzyme- or metal-catalyzed processes (23). $O_2^{\bullet-}$ is an anion molecule that is impermeable to membranes such as the inner membrane of the mitochondria. However, anion channels have also been shown to be able to facilitate the $O_2^{\bullet-}$ transport across the cell or mitochondrial membrane (24,25). $O_2^{\bullet-}$ tends to spontaneously dismute to hydrogen peroxide, particularly when pH is about 4.7 (26). $O_2^{\bullet-}$ can also act as both reductant and an oxidant (27), thus expanding its reactions with many biological molecules such as oxidation of both ascorbic acid and α-tocopherol (28), and reductions of both ketones (29) and metal cations (30).

The superoxide radical formed *in vivo*, either functionally or accidentally, is disposed of by enzyme superoxide dismutase (SOD) discovered in 1969 by McCord and Fridovich (31) and described also in insects (32). This reaction runs instantly and leads to the formation of H_2O_2. In the proximity of iron cation (Fe^{3+}), the $O_2^{\bullet-}$ is oxidized and gives rise to Fe^{2+} cation, which can subsequently react with H_2O_2 in Fenton's reaction to create dangerous $OH^{\bullet-}$ radical.

In the Haber-Weiss reaction, the $O_2^{\bullet-}$ alone reacts with H_2O_2 and again, the $OH^{\bullet-}$ is formed (33). Thus, the uncontrollable presence of $O_2^{\bullet-}$ in the cell is responsible (directly or via intermediates) for further generation of ROS and degradation of macromolecules.

Superoxide radical also plays a key role in adaptive responses which has evolved in many organisms to protect the cellular environment from oxidative stress (see section 2.1). In bacteria, where this process is well described, $O_2^{\bullet-}$ oxidizes transcription factor SoxR which activates transcription of the *SoxS* gene. Intensified expression of the *SoxS* results in increased levels of the specific mRNA and SoxS protein which, in turn, activates the expression of target genes for superoxide dismutase, endonuclease or glucose-6-phosphate dehydrogenase which all have protective functions in the cell (34).

1.2 Hydrogen peroxide – *"Propagator and Messenger"*

Compared to $O_2^{\bullet-}$, H_2O_2 is a stronger oxidant, and is much more permeable to cells or mitochondrial membrane by passive diffusion. The biggest threat posed by H_2O_2 consists in its oxidative features when reacting with $O_2^{\bullet-}$ (Haber-Weiss reaction) or with Fe^{2+}/Cu^{2+} ions (Fenton's reaction). Both elicit generation of dangerous $OH^{\bullet-}$ radical (see below). High concentrations of H_2O_2 (mM level) can cause severe damage on proteins or DNA, as well initiate lipid peroxidation (33).

As in vertebrates, hydrogen peroxide is eliminated by several enzymes with peroxidase activities in insects. The prime role is played by catalase (CAT) that is mainly located in peroxisomes and its main function is to decompose the reactive H_2O_2 into water and oxygen (32). CAT tends to reduce small peroxides such as H_2O_2, but has no effect on larger molecules such as lipid hydroperoxides. Another enzyme, the ascorbate peroxidase (AsPx), scavenges hydrogen peroxide (ascorbic acid + H_2O_2 - dehydroascorbic acid + $2H_2O$) at low concentrations which are not normally scavenged by CAT (which has a high Km) (35). Insects also possess several glutathione S-transferases (GSTs), a diverse family of detoxification enzymes, part of which has peroxidase-like activity (GSTPx) (36-38). GSTPx is effective in targeting hydroperoxides (ROOH+2GSH – ROH + H_2O + GSSG) but is unreactive toward hydrogen peroxide.

Hydrogen peroxide has been shown to be involved in cell signaling, most likely as a second messenger (39). Different concentrations of H_2O_2 affect repression of various genes involved in the T cell response, mitochondrial function, growth arrest of the cell or iron metabolism (40). Similarly as was shown for transcription factor SoxR and its role in adaptive response to OS in bacteria, H_2O_2 exclusively regulates transcription activator OxyR which

exists in oxidized and reduced forms with only the oxidized form activating transcription of *OxyR* regulon genes (41). Products of these genes include antioxidant enzymes CAT, SOD or glutathione reductase (GR) (42). Besides regulation of antioxidant potential within the cell, some organisms also regulate permeability of membrane to avoid uptake of ROS which are formed out of the cell. The adaptation of yeast cells to H_2O_2 challenges rapidly modulates the expression of genes encoding enzymes involved in estrogen and lipid catabolism, leading to alterations of membrane lipid composition (43).

1.3 Hydroxyl radical – *"Executor"*

The attack of a very strong oxidant, such as hydroxyl radical, to biological systems can cause extensive cellular oxidations (44). As mentioned above, there are two main ways of $OH^{\bullet-}$formation from hydrogen peroxide – through Haber-Weiss or Fenton's reaction. Similar to $O_2^{\bullet-}$, the $OH^{\bullet-}$ is also a short-lived molecule (10^{-9} s in cells) and is impermeable to membranes. However, it possesses a high reactivity and thus can react with any molecules in its vicinity at diffusion-limited rates (33,45). This features make $OH^{\bullet-}$ the most dangerous oxygen-radical which can initiate the tremendous chain-reaction in proteins or lipids almost everywhere within the organism (46-48).

There is no enzymatic degradation of the $OH^{\bullet-}$ molecule. However, $OH^{\bullet-}$ as well as $O_2^{\bullet-}$, peroxyl and alkoxyl radicals react readily with antioxidant scavengers such as reduced glutathione (GSH), ascorbic acid (AsA), α-tocopherol or carotenoids (49-51) most of which have also been found in insects (52-54). GSH is the major cell antioxidant and takes part in several protective roles (55): (i) GSH is a cofactor of several detoxifying enzymes against oxidative stress, e.g. glutathione peroxidase (GPx), GST and others; (ii) GSH scavenges hydroxyl radical and singlet oxygen directly, detoxifying hydrogen peroxide and lipid peroxides by the catalytic action of AsPx or GST and (iii) GSH is able to regenerate other antioxidants, vitamins C and E, back to their active forms. GSH together with AsA play key roles in glutathione-ascorbic acid redox cycle in insects (56). In response to increased ROS formation, AsA decomposes H_2O_2 by enzyme AsPx and is thus oxidized to dehydroascorbate (DHAs) (35). Enzyme dehydroascorbate reductase (DHAsR) regenerates AsA from DHAs utilizing the GSH molecule which is oxidized to GSSG (57). GSSG can be recycled to GSH in a NADPH-dependent reaction catalyzed by GR. Activity of each of the enzymes involved in this cycle together with the availability of NADPH and AsA contribute to intracellular GSH/GSSG ratio and thus to the redox status within the cell which is important for regulation of oxidative stress.

2. Oxidative stress

2.1 Oxidative stress and "redox state" homeostasis

The harmful effect of ROS causing potential biological damage is termed oxidative stress (OS) and occurs in biological systems characterized by an overproduction of ROS and/or by a deficiency of enzymatic and non-enzymatic antioxidants. Uncontrolled excess of ROS can damage cellular lipids, proteins, or DNA inhibiting their normal functions. The peroxidation of lipids and the carbonylation of proteins are typical products of excessive oxidation. The variety of lipids and the random nature of ROS reactions can lead to many products, including malondialdehyde (MDA) and 4-hydroxynonenal (4-HNE), which are primarily taken as an indicators of cell membrane damage or apoptosis (58,59). The content of carbonyl groups is commonly used marker of protein oxidation (60). Carbonyls are formed from the amino groups in the side chains of lysine, proline, arginine, and histidine that are exposed to H_2O_2 or O_2 in the presence of redox cycling cations such as Fe^{2+} or Cu^{2+} resulting in Fenton reactions generating hydroxyl and alkoxyl (or peroxyl) radicals (61).

On the other hand, OS can be elicited in (or out of) cells as a part of normal physiological functions such as immune response. The stimulation of immune response may involve production of superoxide and HClO (hypochlorous acid, very strong physiological oxidant) by NADH oxidase and myeloperoxidase in activated phagocytes (62,63). In such case, $O_2^{\bullet-}$ and mainly HClO act like a natural physiological defender against pathogenic microorganisms entering the organism. Quinone compounds or molecules containing a metallic cation (Fe, Cu) may promote $O_2^{\bullet-}$ formation because of the ability to easily give the electron to molecular oxygen (64,65). Both monoamine- and xanthine-oxidases produce H_2O_2 as a by-product during the reactions with monoamines and hypoxanthine (66,67).

The delicate balance between beneficial and harmful effects of free radicals is a very important aspect of living organisms and is achieved by mechanisms called "redox regulation". This process protects living organisms from OS and maintains "redox homeostasis" by controlling the redox status *in vivo* (68). The intracellular "redox homeostasis" is substantiated primarily by GSH and thioredoxin recycling systems. The glutathione couple (2GSH/GSSG) represents the major cellular redox buffer with concentrations up to 10mM in cytosol and is therefore a representative indicator for the redox environment of the cell (69). Thioredoxins (Trx) are small proteins which act as antioxidants by facilitating the reduction of other proteins by cysteine thiol-disulfide exchange and thus are critical for redox regulation of protein function and signaling via thiol redox control. A

growing number of transcription factors including the NF-kB or the Ref-1-dependent AP1 require Trx reduction for DNA binding (70). The high ratios of GSH/GSSG and Trx_{red}/Trx_{ox} are maintained by the activity of GR (see section 1.3) and Trx reductase (TrxR), respectively. However, Kanzok et al. (71) found that the TrxR substitutes for GR in *Drosophila* which points out the importance of this redox-regulating system in insects.

2.2 Biomarkers of oxidative stress

When evaluating OS in a living system, one can analyze and measure plenty of so called oxidative stress biomarkers. Usage of appropriate biomarker should be carefully considered prior to each experiment according to the research interest. In this work, various insect tissues (hemolymph, central nervous system, midgut) were examined in an effort to determine and quantify impact of OS in organism by using different time periods of exposure to various stressors. Those issues were taken into account when exploring antioxidant effects of neuropeptides from AKH family (AKHs, see section 4) in which the mechanism of acting and signal transduction under OS conditions is unknown but their undisputable role in antioxidant response is apparent. For this reason, a wide scale of different OS markers were used to explore this phenomenon according to following criteria:

1) Antioxidant molecules with direct detoxification effect towards free radicals and peroxides. This includes quantification of reduced glutathione or expressing of GSH/GSSG ratio which informs more about "redox state" in the cells or tissue (72) (see sections 1.3). Another biomarker, a total antioxidant activity assay (TAA), relies on antioxidant capacity of low molecular weight antioxidants using vitamin E analog Trolox as a standard (73). Advantage of these markers consists in their relatively easy interpretation under the OS conditions.

2) Antioxidant enzymes which are able to decompose or detoxify ROS and toxic products of oxidation. These include above described enzymes CAT, SOD, GSTs and AsPx (see sections 1.1 and 1.2). Principle characteristic of these biomarkers is their specificity and affinity to target substrate. Gene expressions and activities of these enzymes can vary with respect to intensity of OS, cell compartment and exposure period to stressor.

3) Products of oxidation derived from damaged or disrupted macromolecules like lipids, proteins or DNA following the OS induction. Some of these biomarkers, namely protein carbonyls (60) and lipid peroxides (58), are quite stable derivatives, with non-specific tissue formation pattern and thus can be quantified in relatively large range of stressing period in most tissues exposed to OS.

4) Direct analysis of reactive oxygen species is another measure of the impact of OS and is well established in biological systems, however, methods involved often require complex and costly devices with certain difficulties when optimizing for *in vivo* experiments. Therefore, and also for other reasons, this approach was not used in this study.

3. Induction of OS in insects: an experimental approach

The various stimuli leading to creation of OS conditions in living organisms constitute a highly diverse group of natural and artificial compounds as well as exo- and endogenous sources. Environmental generators of OS usually are industrial pollution, solar radiation or traffic exhaust (74). Experimentally, OS is usually elicited by many stimuli including hyperoxia (75), dietary supplementation with transition metals (76,77), herbicides or insecticides with oxidizing potential (e.g. paraquat, endosulfan, malathion, see below), both heat shock (78) and cold exposure (79), or by stimulation with ROS-precursors, like hydrogen peroxide (80).

When working with insects, the choice of appropriate OS elicitor depends on several factors like 1) developmental stage (larvae, adult), 2) ecological and evolutionary specialization (herbivore, carnivore, piercing-sucking apparatus etc.), 3) connection with mankind (pest, disease vector, model for basic research) and others. These factors determine 1) kind of stressor to be applied (insecticide, herbicide, natural allelochemical), 2) way of application (oral, injection, topically through cuticule), 3) dose (pharmacological, physiological) or 4) duration of OS exposure. In our research, we have used four OS elicitors: 1) paraquat (a herbicide) and insecticides endosulfan and malathion for experiments on the firebug *Pyrrhocoris apterus* (see paper 2 and 3) and tannic acid (plant polyphenolic compound) for experiments on the cotton leafworm *Spodoptera littoralis* larvae (see paper 1).

3.1 Paraquat

Paraquat (1,1'-dimethyl-4,4'-bipyridilium, PQ) is a potent herbicide which undergoes a cyclic redox reaction with oxygen to produce superoxide radicals, singlet oxygen, hydroxyl radicals, hydrogen peroxide, lipid peroxides and disulfides (81-83). Most authorities agree that free radical pathology is the most likely mechanism by which PQ is cytotoxic (81,85). All these free radicals formed after PQ exposure are capable of initiating the peroxidation of membrane lipids, causing tissue damage or even death (86). In the presence of NADPH, PQ is reduced by microsomal NADPH-cytochrome reductase. The reduction of lipid peroxides by GPx or GSTPx requires GSH and leads to GSSG formation which is regenerated by enzyme

GR. Because the reduction of GSSG by GR is coupled with NADPH oxidation, it seems that the availability of NADPH is essential for PQ detoxification, and that the critical depletion of NADPH may render the cell more susceptible to lipid peroxidation.

PQ toxicity mediated by free radicals can be moderated by several cellular defense mechanisms, including SOD, CAT, GPx, vitamin E, and GSH (84,85). The toxicity of PQ was demonstrated in birds, mammals, fish, as well as in aquatic and terrestrial invertebrates and plants (86). Within invertebrates – earthworms, mites, honey bees, two species of springtails and water crustaceans and mollusks show varying degrees of sensitivity to PQ (85,87,88). Recently, PQ treatment was found to be responsible for development of oxidative stress in the colorado potato beetle *Leptinotarsa decemlineata* (1) and in fruitfly *Drosophila melanogaster* (89,90). In the firebug *P. apterus*, paraquat injection (40 pmols) affected several OS markers and processes as follows (11,91): it significantly increased protein carbonylation and decreased GSH level in hemolymph; it shortened survival rate of both males and females; egg laying and consequent hatching was suppressed in females; and finally, PQ decreased fluidity and increased microviscosity of membrane fractions from brain tissue.

3.2 Tannic acid

Plant phenolic compounds, particularly flavanoids and tannins, have long been associated with plant defense against herbivores (92). Both autoxidation and enzyme catalyzed oxidations of phenolic compounds produce semiquinone radicals and quinones. Toxic phenoxyl radicals are formed via oxidative processes owing to their ability to initiate free radical chain reactions in the membrane and the propensity to cross-link with a variety of molecules (93,94).

The midgut of insect herbivores is a highly oxidizing environment. Hence, diet supplementation of lepidopteran larvae *Helicoverpa zea* and *S. littoralis* with phenolic acids was found to increase various indicators of oxidative stress in gut tissues (95,96). Redox-active iron could potentially promote tannin oxidation when oxygen levels are limiting (97). Almost any oxidation of phenolics can result in the generation of superoxide anion radicals because the reactive semiquinone can donate an electron to molecular oxygen. The superoxide anion generated in this manner can further lead to the generation of additional radical species, including hydroxyl radicals. Thus, the propensity of phenolics to generate radicals depends on whether they are ionized or oxidized. The oxidation and ionization of the phenolics depend on their phenolic structure, the physicochemical conditions under which the

reactions take place, including hydrogen ion (pH) and electron (Eh, or redox potential) availability, and the concentration of antioxidant enzymes as well as nonenzymatic oxidants and reductants (98).

Toxic and oxidizing effects of tannic acid (TA) ingested in the diet were described in several insect species previously (98,99). In *S. littoralis* larvae of last developmental instar, the highest ROS formation and most intensive antioxidant defense were found to be allocated to different gut compartments (96). In foregut content, increase in total peroxides and superoxide radical levels was recorded whereas substantial increase in activities of antioxidant enzymes CAT, SOD and AsPx was observed in midgut tissue after 5% TA feeding. Moreover, increased protein carbonylation and decreased GSH level with decreased GST activity as well as increased CAT and SOD gene expression was described later in the midgut tissue (100). These results indicate interesting feeding strategy by utilizing ROS: strong oxidizing milieu of foregut tissue supports the deployment of semiquinones and oxygen radicals after TA feeding which could partially serves as a defense mechanism against pathogens ingested by natural way and partially facilitates digestion of proteins (101). Increased formation of ROS is then dropped abruptly when the food bolus passed from the foregut to the midgut (where the nutrients are absorbed and utilized), apparently due to high activities of the antioxidant enzymes.

3.3 Oxidizing insecticides: malathion and endosulfan

The insecticides malathion and endosulfan, used in a part of this study, are applied against a number of insect pests and mites in agriculture, greenhouses and gardens, as well as for the public vector control. Despite their high toxicity, hazard of their use and restrictions for their usage, they are still employed world-wide and particularly in developing countries. Both insecticides were also clearly proven to elicit OS (see also below).

Malathion is one of the most often used organophosphate insecticide in the world. The primary toxicity of malathion consists in inhibition of enzyme acetylcholine esterase (AChE). This toxicity is manifested after bioactivation of malathion by cytochrome P450 enzymes, which create the active metabolite malaoxon (102,103). Insects, unlike mammals, lack enzyme carboxyl esterase which can detoxify malathion as a substrate; this fact should determine selectivity of this organophosphate towards insects (104). Malathion poisoning can elicit oxidative stress in humans by increasing lipid peroxidation, decrease in GSH level and increase in activities of CAT and SOD antioxidant enzymes (105). In larvae of the wax-moth

Galleria mellonella, malathion application increased lipid peroxidation and SOD activity while decreased GSH level and activity of AChE (106).

Endosulfan is an insecticide with contact and gut action that belongs to a group of organochlorine cyclodiene pesticides. It acts as a neurotoxin (in both insects and mammals) by inhibiting GABA receptors at synapses, Ca^{2+} and Mg^{2+} ATPase, and AChE, and also works also as an endocrine disruptor. In particular, endosulfan has a relatively reactive cyclic sulfite diester group and could be metabolized to endosulfan sulfate (shows similar acute toxicity to the parent compound). Endosulfan-induced oxidative stress was evidenced in yeasts (107), plants (108) and humans (109), mostly by increase in lipid peroxidation products formation and in the activity of antioxidant enzymes and by depletion of antioxidant molecules.

In *P. apterus*, injection of either endosulfan (200 and 250 ng) or malathion (300 and 450 ng) did increase CAT activity and decreased GSH level in hemolymph 3 hours after the treatment. Also the carbon dioxide production was increased in the bugs exposed to both insecticides when compared to control bugs. Topical application of malathion (500 and 900 ng) and endosulfan (450 and 1100 ng) increased bug mortality substantially evidencing ability of these insecticides to penetrate through the cuticle and to act as contact agents as well (3).

4. Insect adipokinetic hormones and their role in oxidative stress

4.1 Introduction to AKHs and energy metabolism

Insect metabolism and especially its energetic part is controlled by adipokinetic hormones (AKHs), small peptides composed mostly from 8 to 10 amino acids, which are synthesized, stored and released by neurosecretory cells from the *corpora cardiaca* (CC), a neuroendocrine gland connected with the insect brain. Generally, AKHs behave as typical stress hormones – they stimulate catabolic reactions by mobilizing lipids (therefore adipo-kinetic), carbohydrates (since trehalose is a predominating form of sugars utilized by insect, they are sometimes called hypertrehalosemic hormones, HrTHs) or certain amino acids, making energy more available, while inhibiting synthetic reactions. Using this strategy they direct the 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 (110).

These hormones have been isolated from representatives of many insect orders (111) and to date, more than 40 insect AKHs have been characterized. All AKHs possess a pyroglutamate residue blocking the N-terminus and an amide group blocking the C-terminus

(except for the AKH of butterfly *Vanessa cardui* with C-terminally extended) (112). The amino acids tryptophan and glycine are at positions 8 and 9 (when present); in addition to tryptophan the molecule contains at least one more aromatic amino acid, most commonly phenylalanine at position 4.

Receptors for AKH (AKHRs) are G protein-coupled and are, structurally and evolutionary, related to the gonadotropin-releasing hormone receptors (GnRH-Rs) from vertebrates (113). Recently, the AKHRs of *Bombyx mori, D. melanogaster* (113), *Periplaneta americana* (114) and *Anopheles gambiae* (115) have been cloned and many others have been deduced from their genetic sequences (116). A fat body is the main target of AKH actions where the signal transduction is well described (117,118). Generally, after binding to G protein-coupled receptor in cellular membrane, AKH can trigger two different pathways leading either to lipid, sugar or amino acid reserves mobilization. In sugar metabolism, G protein-activated phospholipase C initiates formation of inositol 1,4,5-triphosphate (IP3) and/or dyacylglycerols (DAG) from membrane lipids. Either IP3 or DAG can then activate, in presence of intracellular Ca^{2+} ions, phosphorylase kinase or protein kinase C, respectively. This finally leads to phosphorylation of glycogen phosphorylase and production of glucose/trehalose molecules (119,120). In lipid metabolism, G-protein activated adenylate cyclase results in an increase of intracellular cAMP and Ca^{2+} levels. Cyclic AMP stimulates lipase activity, most likely via activation of protein kinase A, leading to fatty acid production from triacylglycerol (TAG) reserves (119,121). Free fatty acids then undergo β-oxidation and the resulting to the acetyl coenzyme A production. In some insects (certain beetles and dipterans) an alternative lipid pathway is used: acetyl coenzyme A serves for conversion of alanine to proline that serves as an energetic substrate (119).

In this work, two distinct insect species were used as experimental models and some more species were discussed when considering the role of AKHs in stress reactions. Below I briefly list all AKHs and HrTHs that have been studied in those species and used for the experimental treatment. The firebug *P. apterus* possesses two AKHs: an octapeptide Pyrap-AKH which is unique in this species (122), and an octapeptide Peram-CAH-II, first identified in the cocroach *P. americana* (123). In all stress experiments, synthetic Pyrap-AKH was used exclusively for hormonal treatments. In the moth *S. littoralis*, Manse-AKH and Helze-HrTH neuropeptides from AKH family were identified (124), however, only Manse-AKH has been chosen for the stress experiments (see paper 1). Lastly, the Colorado potato beetle *L. decemlineata* possesses two AKHs, Peram-CAH-I and Peram-CAH-II, both originally identified in *P.americana* AKH (125).

4.2 Roles of AKH which are not associated with energy metabolism

Although AKHs mostly activate the energetic mobilization during metabolic stress elicited by flying or walking, other roles of these pleiotropic hormones have also been found in different stress challenges. Stress-induced elevation of the AKH titre occurs in *Schistocerca gregaria* and *P. apterus* challenged with insecticides (2-5), photophase interruption (5), or exposure to constant darkness (126). Moreover, coinjection of pyrethroid insecticide permethrin with Pyrap-AKH increase mortality of *P. apterus* bugs compared with bugs treated with the insecticide alone (2). Recently discovered interaction of AKH with the humoral and cellular immune system in *L. migratoria* can also be regarded as a stress response. The prophenoloxidase (ProPO) cascade in the hemolymph of this locust is activated when laminarin is injected, and this activation is prolonged when Locmi-AKH-I is co-injected with the immunogen (8). The injection of bacterial lipopolysaccharide (LPS) from *Escherichia coli* stimulates the formation of nodules but does not increase the phenoloxidase activity in the haemolymph; on the other hand, co-injection of Locmi-AKH-I and LPS results in formation of a greater number of nodules and also activates the ProPO cascade (5,9). It is suggested that these two immunogens must activate the ProPO cascade by quite distinct pathways, that are probably not based on rapid changes in the energy rich metabolites; although, changes in the lipophorins and the apolipoprotein-III coincident with immune challenge point to a participation of lipids in this process (10).

4.3 Role of AKHs in oxidative stress

In the main part of this work, the attention was focused on the role of AKHs in antioxidant response to OS induced by various elicitors. The results of the last 10-year research on AKH have revealed that oxidative stressors increase the level of those hormones either (or both) in CNS or hemolymph, and that exogenous AKHs reverse the OS status in insect body. These facts indicate a feedback regulation between stressors and AKH action in antioxidant protective mechanisms in insects.

AKH-elevating effect has been reported for the following stressors: the above mentioned herbicide paraquat (PQ, see section 3.1) (1,11), *Galanthus nivalis* agglutinin (GNA) and Cry 3Aa-*Bacillus thuringiensis* toxin (1), and insecticides endosulfan and malathion (see section 3.3) (3). In *P. apterus*, 4 hours-exposure to PQ increased the AKHs level in hemolymph about 5 times (11) whereas in *L. decemlineata* the PQ treatment caused 2.7 fold increase of both AKHs in hemolymph compared to non-stressed individuals (1). Both

endosulfan and malathion increased the level of both *Pyrrhocoris* AKHs in the hemolymph of treated bug 2.5 times in 24 hours whereas only slight elevation of this neuropeptide was observed in CNS (3). Unlike the PQ and both insecticides in *P. apterus*, the toxins GNA and Cry 3Aa stimulated elevation of the AKHs in CNS of *L. decemlineata* up to 10 times (1). For the AKHs overview, see section 4.1.

The above mentioned results suggesting involvement of the AKHs in the activation of antioxidative mechanisms are supported by a series of experiments demonstrating direct involvement of AKHs in the modulation of OS biomarker levels. Restoration of GSH level and suppression of protein carbonylation in hemolymph were observed when AKH was co-injected with OS stressors PQ, endosulfan and malathion in *P. apterus* or *L. decemlineata* (1,11,3). Moreover, decrease in CAT activity and increase in total antioxidant capacity of hemolymph were observed after the AKH co-injection with the stressors in *P. apterus*. Involvement of glutathione in AKH-directed antioxidative response is supported by findings that GSH is elevated in hemolymph after the AKH injection in non-stressed individuals.

Role of exogenous AKH in antioxidant response was also demonstrated in the midgut tissue of *S. littoralis* larvae, where 5% TA (supplied in artificial soy-bean diet) was used as the OS elicitor in larvae of 6^{th} (final) instar (see section 3.2) (12). Decreased GST activity after the TA feeding was restored back to control level after AKH was injected while neither CAT nor SOD activities were affected by this hormonal treatment. The AKH-induced increase in GST activity was accompanied by suppression of protein carbonylation which suggests retreat of OS in the midgut tissue. This regeneration of the cells-reducing power corresponded well with rapid decrease in expression of CAT and SOD mRNAs after the AKH treatment. However, the information about a possible pathway or signal transduction through which this AKH-induced antioxidant response is directed is still missing though there are some indications that GSH could take part as a cofactor in enzymatic reactions in this mechanism.

5. Role of other insect hormones in OS

5.1 Glucagon

A certain analogy arises when comparing organisation of hormonal systems in insects and vertebrates: brain (neurosecretory cells) - CC neuroendocrine cells – hemolymph and hypothalamus – pituitary gland (neuro/adenohypophysis) – blood (127). Although there is no apparent structural similarity between AKHs and any vertebrate hypothalamus-pituitary

hormones, AKHs resemble glucagon, a peptidic hormone from the α-pancreatic islet cells in vertebrates that is also responsible for mobilization of energy rich metabolites.

In 1980, a glucagon-like peptide was found in *Manduca sexta* hemolymph (128) and despite certain structural similarity to glucagon and some promising results, neither this natural peptide nor the vertebrate glucagon were proven to mobilize energy stores in *M. sexta* (129,130), and thus the role of intrinsic glucagon in insect body was unclear. However recently, the role of glucagon in activation of insect antioxidant protective mechanisms has been suggested (131). Presence of the glucagon-like peptide(s) were clearly documented in the gut and brain tissues of *P. apterus* using monoclonal antibody against porcine glucagon. Glucagon injected into the hemocoel had no effect on mobilization of lipids, the main energetic resources in the firebug, whereas the same dose did reverse paraquat-induced OS status in hemolymph by increasing of the GSH level and decreasing of protein carbonylation. These results are not so surprising because glucagon plays a protective role in OS also in vertebrates. Lu et al. (132) reported that glucagon-mediated signal transduction pathways lead to a down-regulation of hepatic GSH synthesis while promoting its efflux to the blood plasma in rats. In addition, glucagon may also act in part by stimulating the GSH mediated reduction of protein disulfides by the thiol:protein disulfide oxidoreductase as demonstrated in isolated rat hepatic microsomes (133). These findings clearly indicate at least a partial role of glucagon in the activation of antioxidative systems in both vertebrates and insects. In addition, the lack of significant changes of AKH titre in *P. apterus* body after the injection of glucagon suggests that the action of glucagon is AKH independent (131).

5.2 Ecdysteroids

Ecdysteroids are steroid hormones in insects produced primarily by prothoracic glands and partially also by several other tissues (132). They mainly control molting, development, metamorphosis and reproduction, and are also involved in a number of diverse processes. Regarding the role of ecdysteroids in OS, 20-hydroxyecdysone was found to be a potent antioxidant able to minimize the OS impact of paraquat to *P. apterus* (91, paper 5). This ecdysteroid restrained lipid peroxidation and the formation of protein carbonyls, ameliorated changes in microsomal membrane fluidity, enhanced the level of reduced glutathione, and upregulated the activity of γ-glutamyl transpeptidase in the brain. At the organismal level, 20-hydroxyecdysone enhanced survival rate and increased female fertility when injected in PQ-treated individuals.

A protective function during OS was also described for 20 hydroxyecdysone precursor, ecdysone, in *D. melanogaster* (133). Ecdysone enhanced resistance to H_2O_2 by inducing methionine sulfoxide reductase A, an enzyme which catalyzes reduction of oxidized methionine residues. Overexpression of the enzyme was associated with enhanced protection against OS, while its knockdown results in hypersensitivity to OS (134). In vertebrates, steroid hormones estrogens and related compounds possessing a phenolic A-ring, were shown to be involved in the control of OS as well. They inhibited the oxidation of cholesterol and the peroxidation of polyunsaturated fatty acids in the lipoproteins, microsomes, and other components of biological systems (135). All these examples suggest a role of steroid hormones as agents with high antioxidative potential in both insects and vertebrates, however, the precise mechanism of action of these effects in mostly unknown.

5.3 Juvenile hormone (JH)

These terpenoid hormones have two main functions in insect life: as developmental hormones, they prevent premature initiation of insect metamorphosis in juvenile stadia whereas in reproduction, they primarily control gene expression of vitellogenins in adult females. Their role in OS seems to be indirectly mediated through the regulation of biologically active proteins like vitellogenins and/ or transferrin (138,139).

Vitellogenins, which are complex glycol-lipo-protein molecules, primarily serve as energetic and building components for a developing embryo in the insect egg. They are synthesized in specialized cells in the fat body or rarely in ovariols by a complicated hormonally controlled process orchestrated in most insect species by JHs. Vitellogenins protected the bee workers against the oxidative damage of PQ as they were the preferred target of deleterious carbonylation among other hemolymph proteins (140). Also, survival rate was significantly increased in high-vitellogenin level bee phenotypes compared to low-vitellogenin ones after PQ exposure indicating the importance of vitellogenins in OS prevention in insects.

Insect transferrin is known as a transporter of iron that is required for a wide variety of metabolic processes including oxygen transport and electron transfer. Transferrin is supposed to be under control of JH in vitellogenic process, and since free iron ions participate in harmful Fenton’s reaction under OS conditions, the iron metabolism should be well orchestrated in terms of preventing tissues from oxidative damage. Nevertheless, the suppressive effect of JH and its analog methoprene on transferrin function in cockroach

Blaberus discoidalis (138) and in mosquito, *Aedes aegypti* (140), is rather intriguing and not well understood yet.

List of abbreviations

4-HNE – 4-hydroxynonenal
AChE – acetylcholine esterase
AKH – adipokinetic hormone
AKHR – AKH receptor
AP-1 – activator protein 1 (transcription factor)
AsA – ascorbic acid, ascorbate
AsPx – ascorbate peroxidase
CAT – catalase
CC – corpora cardiaca
Cry 3Aa – Cry 3Aa-*Bacillus thuringiensis* toxin
DAG - diacylglycerol
DHAs – dehydroascorbate
DHAsR – dehydroascorbate reductase
GABA – γ-aminobutyric acid
GNA – *Galanthus nivalis* agglutinin
GnRH-R – gonadotropin-releasing hormone receptor
GPx – glutathione peroxidase
GR – glutathione reductase
GSH – reduced glutathione
GSSG – oxidized glutathione
GST – glutathione S-transferase
GSTPx – glutathione S-transferase with peroxidase activity
H_2O_2 – hydrogen peroxide
HClO – hypochlorous acid
Helze-HrTH – HrTH first identified in *Helicoverpa zea*
HrTH – hypertrehalosemic hormone
IP_3 – inositol 1,4,5-triphosphate
JH – juvenile hormone
Keap1 – Kelch-like ECH-associated protein 1 (stress sensing protein)
Locmi-AKH-I – AKH first identified in *Locusta migratoria*
LPS – lipopolysaccharide
MDA - malondialdehyde
Manse-AKH – AKH first identified in *Manduca sexta*
MAP kinase – mitogen-activated protein kinase
NF-κB – nuclear factor kappa-light-chain-enhancer of activated B cells
Nrf2 – Nuclear factor (erythroid-derived 2)-like 2 (transcription factor)
$O_2^{\bullet-}$- superoxide radical
$OH^{\bullet}$ - hydroxyl radical
OS – oxidative stress
OxyR – hydrogen peroxide-response system (described in *Escherichia coli*)
Peram-CAH-I(II) – cardio-accelerating hormone(s) first identified in *Periplaneta americana*
ProPO – prophenoloxidase
PQ – paraquat
PT pore – permeability transition pore (mitochondrial)
Pyrap-AKH – AKH first identified in *Pyrrhocoris apterus*
ROS – reactive oxygen species
SOD – superoxide dismutase
SoxRS – superoxide-response system (described in *Escherichia coli*)
TA – tannic acid
TAA – total antioxidant assay (a commercially available detection kit)
TAG – triacylglycerol
Trolox – 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (a derivative of vitamine E)
Trx – thioredoxin (Trx_{red} – reduced form, Trx_{ox} – oxidized form)
TrxR – thioredoxin reductase

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Results

Main body of the thesis

Paper 1

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Adipokinetic hormone-induced antioxidant response in *Spodoptera littoralis*

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ABSTRACT

The antioxidative potential of the *Manduca sexta* adipokinetic hormone (Manse-AKH) in the last instar larvae of *Spodoptera littoralis* (Noctuidae, Lepidoptera) was demonstrated after exposure to oxidative stress (OS) elicited by feeding on artificial diet containing tannic acid (TA). Determination of protein carbonyls (PCs) and reduced glutathione (GSH) levels, monitoring of activity of antioxidant enzymes catalase (CAT), superoxide dismutase (SOD) and glutathione-S-transferases (GSTs), as well as measuring of the mRNA expression of CAT and SOD were used as markers of the OS. Injection of the Manse-AKH (5 pmol per individual) reversed the OS status by mitigation of PCs formation and by stimulation of glutathione-S-transferases (GSTs) activity. The CAT and SOD mRNA expression was significantly suppressed after the Manse-AKH injection while activity of these enzymes was not affected. These results indicate that diminishing of OS after the AKH injection might be a result of activation of specific enzymatic pathway possibly at the post-translational level rather than a direct effect on regulation of antioxidant marker genes at the transcriptional level.

Paper 2

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Oxidative stress elicited by insecticides: A role for the adipokinetic hormone

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ABSTRACT

Adipokinetic hormones (AKHs) are insect neuropetides responding to stress situations including oxidative stress. Two insecticides – endosulfan and malathion – were used to elicit oxidative stress conditions in the firebug *Pyrrhocoris apterus*, and the physiological functions of AKHs and their ability to activate protective antioxidative reactions were studied. The insecticide treatments elicited only a slight increase of the AKH level in CNS, but more intensive increase in haemolymph, which indicates an immediate involvement of AKH in the stress response. The treatment also resulted in a significant increase of catalase activity in the bug's body and depletion of the reduced glutathione pool in the haemolymph, however, co-application of the insecticides with the AKH (80 pmol) reduced the effect. It has also been found that co-application of the insecticides with AKH increased significantly the bug mortality compared to that induced by the insecticides alone. This enhanced effect of the insecticides probably resulted from the stimulatory role of AKH on bug metabolism: the carbon dioxide production was increased significantly after the co-treatment by AKH with insecticides compared to insecticide treatment alone. It was hypothesized that the increased metabolic rate could intensify the insecticide action by an accelerated rate of exchange of metabolites accompanied by faster penetration of insecticides into tissues.

Paper 3

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Activation of insect anti-oxidative mechanisms by mammalian glucagon

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ABSTRACT

Resembling the main function of insect adipokinetic hormones (AKHs), the vertebrate hormone glucagon mobilizes energy reserves and participates in the control of glucose level in the blood. Considering the similarities, the effect of porcine glucagon was evaluated in an insect model species, the firebug *Pyrrhocoris apterus*. Using the mouse anti-glucagon antibody, presence of immunoreactive material was demonstrated for the first time in the firebug CNS and gut by ELISA. Mammalian (porcine) glucagon injected into the adult bugs showed no effect on hemolymph lipid level or on the level of AKH in CNS and hemolymph, however, it activated an antioxidant response when oxidative stress was elicited by paraquat, a diquaternary derivative of 4, 4′-bipyridyl. Glucagon elicited the antioxidant response by increasing glutathione and decreasing protein carbonyl levels in hemolymph, decreasing both protein carbonyl and protein nitrotyrosine levels in CNS. Additionally, when co-injected with paraquat, glucagon partially eliminated oxidative stress markers elicited by this redox cycling agent and oxidative stressor. This indicates that glucagon might induce an antioxidant defense in insects, as recently described for AKH. Failure of glucagon to alter AKH level in the bug's body indicates employment of an independent pathway without involving the native AKH.

Supportive publications

Paper 4 (published and defended as part of author's rigorous work)

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Adipokinetic hormone-induced enhancement of antioxidant capacity of *Pyrrhocoris apterus* hemolymph in response to oxidative stress

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Abstract

The *in vivo* effects of oxidative stress on adipokinetic hormone (AKH) titer in short-winged (brachypterous) males of the firebug *Pyrrhocoris apterus* were tested using paraquat (PQ), a bipyridilium herbicide. PQ undergoes a cyclic redox reaction with oxygen during microsomal and electron transfer reactions forming free radicals in the insect body. Oxidative insult (40 pmol PQ) resulted in enhanced protein carbonylation (a biomarker for oxidative stress) and a depletion of glutathione (GSH) pool in the hemolymph. Interestingly, AKH titer was significantly enhanced in hemolymph at 4 h post inoculation of PQ, while its content in CNS (brain with corpora cardiaca) showed non-specific changes in comparable period. Co-injection of AKH with PQ (40 pmol each) reversed these effects by decreasing protein carbonyl formation, increasing reduced GSH levels, and enhancing the total antioxidant capacity of cell free plasma. Our results indicate that there is a positive feedback regulation between an oxidative stressor action and the level of AKH in insect body, and that AKHs might be involved in the activation of antioxidant protection mechanism.

Keywords: Adipokinetic hormone; Antioxidant activity; Oxidative stress; Paraquat

Paper 5 (published and defended as part of author's rigorous work)

Archives of Insect Biochemistry and Physiology 65:114–124 (2007)

20-Hydroxyecdysone Prevents Oxidative Stress Damage in Adult *Pyrrhocoris apterus*

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Injections of 38 pmol paraquat (1,1′-dimethyl-4,4′-bypyridilium) into adult *Pyrrhocoris apterus* (average body weight 29.6 mg in males and 36.9 mg in females) caused a significant elevation of lipid peroxidation and protein carbonylation and a decline of membrane fluidity in the microsomal brain fraction. Another manifestation of oxidative stress was a depletion of the reduced glutathione pool and reduction of the γ-glutamyl transpeptidase activity in the brain extracts. The damaging action of paraquat on the brain was counteracted by simultaneous injection of 1 pmol 20-hydroxyecdysone (20E). 20E restrained lipid peroxidation and the formation of protein carbonyls, ameliorated changes in microsomal membrane fluidity, enhanced the level of reduced glutathione, and upregulated the activity of γ-glutamyl transpeptidase. At the organismic level, 20E curtailed three detrimental effects caused by paraquat injection: the disappearance of a blood protein, the suppression of fecundity and egg hatchability, and the shortening of adult life span. The data showed that 20E provided a systemic antioxidant protection but the significance of endogenous ecdysteroids in the management of oxidative stress remains to be shown. Arch. Insect Biochem. Physiol. 65:114–124, 2007.

Keywords: glutathione; γ-glutamyl transpeptidase; 20-hydroxyecdysone; insect brain; lipid peroxidation; membrane fluidity; oxidative stress; paraquat; protein carbonyls

Paper 6 (published during PhD study)

Insect science

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The effects of selection for early and late reproduction on metabolite pools in *Acanthoscelides obtectus* Say

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Abstract The present study was aimed at revealing the responses of metabolite pools to selection for alternative reproductive schedules in the seed beetle, *Acanthoscelides obtectus* Say (Coleoptera: Chrysomelidae: Bruchinae). The levels of metabolites (free sugars, glycogen, lipids, soluble and hydrophobic proteins) that were determined in virgin females and males at three ages from adult eclosion onwards were compared among the base population (B) and two derived lines that were selected for either early (Y) or late (O) reproduction. The results showed differences in the accumulation of metabolites during pre-adult development, as well as in the pattern of their changes during adult ageing. Generally, in comparison to the B population, the short-lived beetles from the Y line showed increased protein content and reduced carbohydrate and lipid content, whereas the opposite was true for the long-lived beetles from the O line. Females from the O line exhibited slower utilization of energy reserves and a slower increase in protein contents than females from the Y line. Females contained higher levels of free sugars, glycogen and hydrophobic proteins and lower levels of lipids and soluble proteins than males, although the sexual dimorphism was not evenly expressed among lines. Age-specific changes in metabolite contents were slower in females than males. Our findings suggest that trade-offs among capital resources are a physiological basis of early/late fitness trade-offs and point to a conservation of resources that can be used for somatic maintenance.

Key words ageing, energy resources, laboratory evolution, proteins, seed beetle, trade-off

Conclusions

1. Manse-AKH reverses OS status elicited by TA feeding in *S. littoralis* larvae (paper 1)

TA-rich diet (5%) supplied to *S. littoralis* larvae in 6th instar caused significant decrease in GSH level and activity of GST enzymes, and increase in protein carbonylation and mRNA expression of CAT and SOD genes, all after 12 hours of the TA feeding. When Manse-AKH (5 pmol) was injected in the middle of the feeding period, gene expression of CAT and SOD dropped to control level, protein carbonylation was suppressed and GST activity increased. These results suggest at least two possible modes of action of this hormone during the TA-induced OS: either by the control of GSH level within the tissue, which could increase reducing power of the cell by increased GSH/GSSG ratio, or by stimulation of GST enzymes through unknown signaling pathways.

2. Pyrap-AKH enhances antioxidant status and intensifies the effects of insecticides endosulfan and malathion on mortality in *P. apterus* (paper 2)

Injection of either endosulfan (250 ng) or malathion (450 ng), elevated the AKH titre in hemolymph and CNS as well as increased activity of antioxidant enzyme CAT and decreased level of GSH in the same bug 3 hours after the treatment. Exogenous application of Pyrap-AKH considerably decreased CAT activity and increased GSH level under OS elicited by both insecticides. Interestingly, carbon dioxide production and also mortality (caused by topical application or injection of either insecticide) were intensified when AKH was co-applied. These findings indicate a versatile effect of AKH in these processes: AKH could enhance antioxidant status that can help insects to cope with OS induced by the insecticides. On the other hand the AKH action might be counter-productive: AKH-stimulated increase of insect metabolism can intensify the insecticides action by their faster penetration into the tissues.

3. Glucagon mimics role of AKH in antioxidant response in *P. apterus* after PQ treatment (paper 3).

PQ (40 pmol) injected into the firebug *P. apterus* did increase protein carbonylation and decrease GSH level in hemolymph 4 hours after the treatment. Both biomarkers were reversed after co-injection of glucagon (50 pmol) with PQ within 4 hours. This effect is identical to previously described role of AKH in antioxidant response in this species (paper 4) and supports the hypothesis that the main function of the glucagon-like peptides is a role in the antioxidant protection of insect body.

Concluding remarks

Exploring the field of oxidative metabolism and its interactions with antioxidant defense mechanisms has revealed plenty of results and new interesting information till date, but practically as many questions have also come up. In my opinion (and not just mine I guess), the insect is a very good model for investigations related to oxidative stress. Compared to vertebrates, the work on this animal model brings a lot of advantages, such as availability of many species, low costs of keeping a breeding, practically no ethical questions arising from killing the insect individual, short period of development and reproducing, and thus minimal time delay in work, and many others. All these advantages have supported and accelerated further experiments and conclusions, which have been demonstrated on the field of insect endocrinology and have also demonstrated some similarities to human endocrine system. Thus, besides the important contribution to the pest control, the research on insect endocrinology in connection to oxidative stress may bring promising results and consequently a possible treatment for tremendous and incurable human neurodegenerative or immune diseases in the future. I hope this study could help in progress in some of these topics mentioned above.

Curriculum vitae

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EDUCATION

2007 – present	PhD student at the University of South Bohemia, Faculty of Science, specialization: Animal Physiology and Developmental Biology (Thesis title: The role of adipokinetic hormones in oxidative stress. Mentor: Prof. Dalibor Kodrík)
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2002 – 2005	B.Sc. degree: University of South Bohemia, Faculty of Science, specialization: Laboratory Medicine (Thesis title: Differences in cytokine production between isolated PBMC and diluted whole blood. Mentor: Prof. Ladislav Janský)

LIST OF PUBLICATIONS

Večeřa, J., Krishnan, N., Mithöfer, A., Vogel, H., Kodrík, D. Adipokinetic hormone-induced antioxidant response in *Spodoptera littoralis. Comp. Biochem. Physiol. C*., in press

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