

REVIEW

The influence of hormones on the lipid profile in the fat body of insects**M Cerkowniak¹, A Ostachowska¹, M Słocińska², G Rosiński², P Stepnowski³, M Gołębiowski¹**¹Laboratory of Analysis of Natural Compounds, Department of Environmental Analysis, Faculty of Chemistry, University of Gdańsk, ul. Wita Stwosza 63, 80-308 Gdańsk, Poland²Department of Animal Physiology and Development, Adam Mickiewicz University, Umultowska 89, 61-614 Poznań, Poland³Laboratory of Chemical Environmental Risks, Department of Environmental Analysis, Faculty of Chemistry, University of Gdańsk, ul. Wita Stwosza 63, 80-308 Gdańsk, Poland

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Abstract

Peptide hormones play a special role in the neuroendocrine systems of insects and affect a number of physiological processes related to their development, reproduction and behavior. The lipid content in the fat body of insects is closely correlated with the work of the endocrine glands. The lipid profile of the fat body of the *Zophobas atratus* beetle reveals a predominant proportion of triacylglycerols when compared to free fatty acids and other lipid compounds, such as fatty acid esters, fatty alcohols and sterols. Although it may depend on the stage of the insects' development, the disparate impacts of the adipokinetic hormone (AKH) on the lipid content in the fat bodies of the feeding larvae and the non-feeding pupae of *Z. atratus*, may signify the different roles this hormone plays in the indirect control of the insects' metabolism.

Key Words: fat body; adipokinetic hormone; sulfakinins, insects; *Zophobas atratus***Introduction**

The neuroendocrine systems of insects play a special role in the regulation of most of their metabolic

List of abbreviations:

Adipokinetic hormone, AKH; Red Pigment Concentrating Hormone, RPCH; Sulfakinin, SK; programmed cell death, PCD; High Performance Liquid Chromatography with a Laser Light-Scattering Detector, HPLC-LLSD; Gas Chromatography with Flame Ionization Detector, GC-FID; Gas Chromatography Mass Spectrometry, GC-MS; Trimethylchlorosilane, TMCS; *N,O*-bis(trimethylsilyl) trifluoroacetamide, BSTFA; Matrix Assisted Laser Desorption Ionization - Time of Flight, MALDI-TOF; High-Performance Liquid Chromatography/Atmospheric Pressure Chemical Ionization - Mass Spectrometry, HPLC/APCI-MS; High-Performance Liquid Chromatography/Mass Spectrometry, HPLC/MS; Electrospray Ionization Quadrupole Ion Trap Mass Spectrometry, ESI QITMS; Thin-Layer Chromatography, TLC; Phosphatidylethanolamine, PE; Phosphatidylcholine, PC

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processes as well as in their development. Hormonal regulation plays a key role in many processes including molting and metamorphosis, for example the ecdysone and juvenile hormone (Koeppel *et al.*, 1985; Riddiford, 1985; Hutchins, 2003), hemolymph metabolite (proteins, carbohydrates and lipids) homeostasis (sulfakinins; Audsley and Weaver, 2009) as well as in energy metabolism during flight (AKH; van der Horst *et al.*, 1997, Van der Horst *et al.*, 2001; Lorenz and Gäde, 2009). In the 1920s, secretory cells were discovered in the brains of insects which control different processes in different parts of the insect's body. From 1917-1922, pioneering research into the mechanisms which regulate the metamorphosis of the gypsy moth caterpillar (*Lymantria dispar*) was carried out by the Polish entomologist Stefan Kopec (Słocińska, 2009).

There are two types of glands involved in the synthesis and release of peptides in insects: the exocrine and endocrine glands. The exocrine glands secrete compounds on the surface of the insect which serve to protect, either by acting as repellents or as attractants. An example of an attractant are pheromones - which are a complex mixture of chemicals (Martins and Ramalho-Ortigão, 2012; Ottaviani, 2014). Examples of endocrine glands are - among others - the *prothorax*, *corpora allata* which produce the juvenile hormone responsible for the process of transforming insects and the *corpora cardiaca*, which are adjacent to the heart and brain

and which secrete hormones that stimulate the mobilization of lipids in locusts (Biej-Bijenko, 1976).

The fat body of insects is made up of trophic tissue and is rich in triacylglycerols, free fatty acids and cholesterol. The fat body is functionally equivalent to the liver and adipose tissue in mammals. A series of transformations of the intermediary metabolism of insects takes place in the fat body, which are under the strict control of hormones secreted by the neuroendocrine system. Metabolites such as carbohydrates, proteins and fats are stored in the fat body. In insects, these compounds are the source of energy for activities such as metamorphosis, flight and egg formation. (Fernando-Warnakulasuriya *et al.*, 1988; van der Horst *et al.*, 1997; Ryan and van der Horst, 2000; Ziegler and Ibrahim, 2001; Guedes *et al.*, 2006; Arrese and Soulages, 2010; Snart *et al.*, 2015). During the changes which occur in the body of the insect, the delivery of large amounts of energy are required and processes occur in the fat body which release trehalose, diacylglyceride and the insect hemolymph protein.. The basic lipid composition of certain selected species of insects is already quite well known. But by using modern chromatographic methods, *e.g.*, gas chromatography or liquid chromatography, the contribution of individual groups of compounds in the lipids can be successfully determined. In many species of insect, a significant amount of free fatty acids and their esters and hydrocarbons or alcohols have been discovered. However, the exact impact of various environmental factors on the lipid profile in the fat body of insects is still not fully known.

The role of the adipokinetic and sulfakinin hormones

The adipokinetic hormone controls - among other things - the mobilization of reserves of energy to

the muscles of wings by releasing trehalose, diacylglyceride and proline to the hemolymph. AKH is a hormone pleiotropic, which not only affects the locomotor activity of the insect, but also regulates the synthesis of RNA, proteins and free fatty acids in the fat body, the activity of the heart and the propagation of the insect (van der Horst and Ryan, 2012).

The first peptide (pELNFSPGWa) belonging to the family of AKH - the red pigment concentrating hormone (RPCH) was isolated from a pink shrimp (*Pandalus borealis*) in 1972 (Fernlund and Josefsson, 1972). Currently, 40 different types of AKH peptides are known (Gäde and Marco, 2009). Their construction is made up of 8 - 10 amino acid residues. Pyroglutamic acid residue is located at the end of the *N*-terminus of the amino acid chain and at the *C*-terminus there is carboxamide residue (van der Horst and Ryan, 2012). As a result of the binding of AKH peptides with G protein receptors located in the fat body, there is a mobilization of carbohydrates. In Table 1, the structure of the AKH family of peptides is shown.

Another important group of peptide hormones in insects are sulfakinins (SK). In their structure, these myotropic neuropeptides have sulfated residues of tyrosine. By using high performance liquid chromatography (HPLC), the peptide family of sulfakinins was isolated from an extract of cockroach *L. maderace* (peptide Leum-SK-1) (Nachman, 1986). Sulfakinins were found not only in cockroaches (Blattodea), including the american cockroach (*Periplaneta americana*) and the madeira cockroach (*Leucophea maderace*) and the housefly (Diptera), including the bluebottle fly (*Calliphora vomitoria*), the australian sheep blowfly (*Lucilia cuprina*), the common fruit fly (*Drosophila melanogaster*) and the grey flesh fly (*Neobellieria bullata*).

Table 1 Example structure of peptides of the AKH family in various insect species

Insects	Peptide sequence	References
<i>Tenebrio molitor</i>	pQLNFSPNWa	(Gäde and Rosiński, 1990)
<i>Zophobas rugipes</i>	pQLNFSPNWa	(Gäde and Rosiński, 1990)
<i>Leptinotarsa decemlineata</i>	pQLTFTPNWa	(Gäde, 1989)
<i>Onitis aygulus</i>	pQYNFSTGWa	(Gäde, 1997)
<i>Pachnoda marginata</i>	pQLNYSFDWa	(Gäde, 1989)
<i>Coccinella septempunctata</i>	pQLNFTPNWa	(Neupert, 2007)
<i>Cheilomenes luncata</i>	pQLNFTPNWa	(Neupert, 2007)
<i>Locusta migratoria</i>	pQLNFTPNWGTa	(Stone <i>et al.</i> , 1976; Bogerd <i>et al.</i> , 1995)
<i>Schistocerca gregaria</i>	pQLNFTPNWGTa	(Hekimi <i>et al.</i> , 1989; Schulz-Aellen <i>et al.</i> , 1989)
<i>Melanoplus sanguinipes</i>	pQLNFTPNWGTa	(Taub-Montemayor <i>et al.</i> , 1997)
<i>Tribolium castaneum</i>	pQLNFSTDWa	(Amare and Sweedler, 2007)
<i>Manduca sexta</i>	pQLTFTSSWGa	(Ziegler <i>et al.</i> , 1985; Bradfield and Keeley, 1989)
<i>Heliothis zea</i>	pQLTFTSSWGa	(Jaffe <i>et al.</i> , 1986)
<i>Bombyx mori</i>	pQLTFTSSWGa	(Ishibashi <i>et al.</i> , 1992)
<i>Apis mellifera</i>	pQLTFTSSWGa	(Lorenz <i>et al.</i> , 1999)

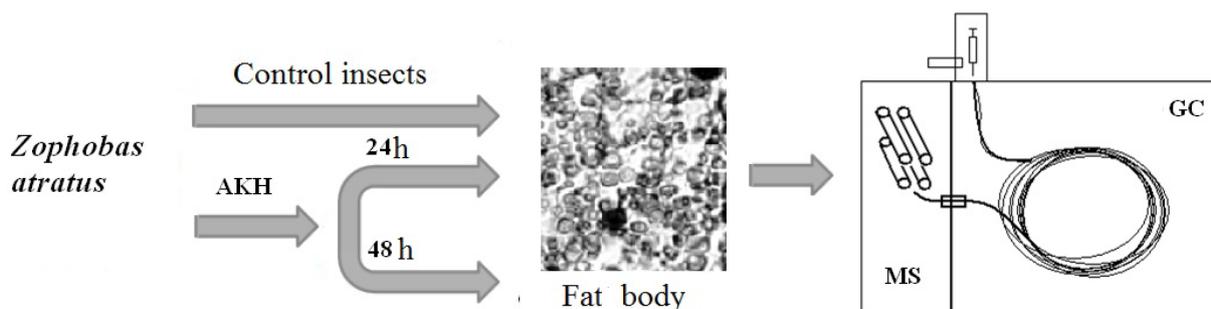


Fig. 1 Overall diagram of the analysis of fat body lipids in *Zophobas atratus* (Gołębiowski *et al.*, 2014). Extracts from the fat bodies were prepared at the Department of Physiology and Biology Adam Mickiewicz University in Poznan.

Sulfakinins play a special role in insects' process of eating by modulating the muscle contractions of the intestines and heart. Sulfakinins also influence the inhibition of food intake in cockroaches and stimulate the secretion of digestive juices in the great scallop (*Pecten maximus*) and the red palm weevil (*Rhynchophorus ferrugineus*) (Schoofs and Nachman, 2006). Sulfakinins control the amount of storage energy and also the composition and amount of free fatty acids and cholesterol, thus affecting the maintenance of homeostasis. The physiological properties of sulfakinins have a functional similarity to gastrin and cholecystokinin which occur in vertebrates (Audsley, 2009).

Methods of analysis

Various methods of extractions are used in the isolation of compounds (analytes) from the matrix. The most popular method for the extraction of lipids is liquid extraction. Chloroform and hexane are used to isolate medium polar and non-polar compounds (Nelson *et al.*, 1999; Buckner *et al.*, 2009). However, more often dichloromethane and petroleum ether are used (Cerkowniak *et al.*, 2013).

One of the most important stages in the analysis of lipid composition is the group analysis. Two common techniques that have been previously used for the separation of particular groups of compounds are thin layer chromatography (Mardaus and Buckner 1997) and high performance liquid chromatography (Cerkowniak *et al.*, 2013). Increasingly popular is high performance liquid chromatography using a laser light-scattering detector (HPLC-LLSD) (Gołębiowski 2012, Cerkowniak *et al.* 2013; Gołębiowski *et al.*, 2013a). For the specific analysis of lipids extracted from the fat or glandular secretions of insects, either gas chromatography with a flame ionization detector (GC-FID) or gas chromatography combined with mass spectrometry (GC-MS) can be used (Durak and Kalender, 2007; Gołębiowski *et al.*, 2013b). Mass spectra can be obtained using a mass spectrometer as a detector, from which test compounds can be identified. For the purposes of quantitative analysis, an internal standard method is normally used. In this method, an internal standard is added to a predetermined amount of sample,

whose retention time will differ from all of the examined analytes. The relationship between the ratio of the concentrations and the ratio of detector response of the test compound and the internal standard is determined.

Figure 1 shows the schematic procedure for the determination of the lipid content in the fat body of the giant mealworm beetle (*Z. stratus*) (Gołębiowski *et al.*, 2014). The extracts were separated into individual groups of compounds in the normal phase using high performance liquid chromatography with HPLC-LLSD. From the fractions, a sufficient quantity of lipids was collected, evaporated to dryness, added to the internal standard, and then silylated with *N,O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA) and trimethylchlorosilane (TMCS). Free fatty acids can be analyzed as trimethylsilyl derivatives or as corresponding fatty acid methyl esters (Gołębiowski *et al.*, 2014; Radzik-Rant *et al.*, 2014). Derived lipids and native organic compounds can be analyzed

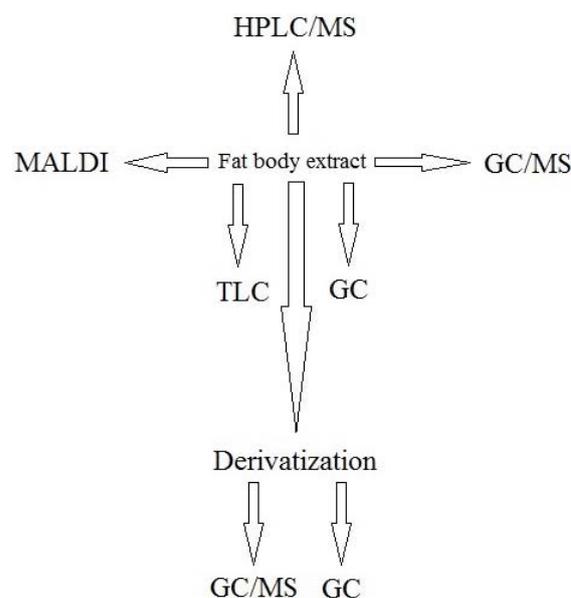


Fig. 2 Techniques used in the analysis of the fat body.

Table 2 Examples of the use of analytical techniques in the analysis of fat body composition

Insects/ Extraction solvent/ Compounds/ Reference	Techniques
11 bumblebee species, e.g.: <i>Bombus terrestris</i> , <i>B. lucorum</i> CHCl ₃ /CH ₃ OH (1:1, v/v) Triacylglycerols. The TGs consisted predominantly of FAs with an even number of carbons, mostly 18 or 16. (Kofronova <i>et al.</i> , 2009)	HPLC/APCI-MS Columns: Two stainless steel Nova-Pak C18 columns (300mm×3.9mm, 150mm×3.9mm, a particle size of 4 μm) connected in a series. Phase: Acetonitrile (A) and 2-propanol (B) The gradient program was: 0 min: 100% of A, flow rate 1 mL/min; 108 min: 30% of A, 70% of B, 1 mL/min; 150 min: 5% of A, 95% of B, 0.5 mL/min; 165 min: 5% of A, 95% of B, 0.5 mL/min, 177-100% of A, 0.5 mL/min; 180 min: 100% of A, 1 mL/min. MALDI-MS An acceleration voltage of 20 kV and a 200 ns extraction pulse. Desorption and ionization were achieved using a nitrogen UV laser (337.1 nm, with a 4 ns pulse of 300 μJ, and the maximum frequency of 20 Hz) with the laser power adjusted to 50-60%.
<i>Bombus lucorum</i> , <i>B. terrestris</i> , <i>B. lapidarius</i> , <i>B. hypnorum</i> , <i>B. hortorum</i> , <i>B. confuses</i> CHCl ₃ /CH ₃ OH (1:1, v/v) Triacylglycerols. The most abundant fatty acids in bumblebees TAGs contained 18 or 16 carbon atoms. (Cvacka <i>et al.</i> , 2006)	GC Column: DB-WAX (30m×0.25 mm, 0.25μm). Conditions: 140 °C (0 min), then 5 °C/min to 230 °C (30 min). HPLC/MS Column: 250mm×4mm packed with Biospher PSI 100 C18, 5 μm. Phase: Acetonitrile (A) and 2-propanol/acetonitrile (3:1, v/v) (B). The linear gradient from 25% of B to 100% of B in 30 min, followed by 5 min at 100% of B.
<i>Pyrhocoris apterus</i> Folch procedure Phospholipids. Two phospholipid classes, phosphatidylethanolamine (PE) and phosphatidylcholine (PC), represent more than 80% of total phospholipids. (Hodkova <i>et al.</i> , 2002)	ESI QITMS Positive ESI/MS and MS ² and/or MS ³ spectra were recorded at 4.5 kV, with capillary voltage 8 V and capillary temperature 190°C. Negative ESI spectra were recorded at 4.5 kV, with capillary voltage -21.5 V and capillary temperature at 190°C.
<i>Zophobas atratus</i> CHCl ₃ /CH ₃ OH (1:1, v/v) Total and phospholipid fatty acid composition. The quantitatively major components were 16:0, 16:1, 18:0, 18:1, and 18:2n-6. (Howard and Stanley-Samuelson, 1996)	GC Column: Supelcowax 10 capillary column (30m×0.25 mm, 0.25μm). Conditions: 2°C/min from 150 to 250°C with an initial 2 min hold. GC-MS Column: Supelcowax 10 capillary column (30m×0.25 mm, 0.25μm). Conditions: 1°C per min from 170 to 220°C.
<i>Rhodnius prolixus</i> Bligh and Dyer procedure Triacylglycerols. (Pontes <i>et al.</i> , 2008)	TLC thin-layer chromatography Developing solvent: hepane-etyl ether-acetic acid (60:40:1 v/v/v). Visualization: 10% cupric sulfate (w/v) in 8% phosphoric acid (v/v) for 30 s.
<i>Bombus pratorum</i> , <i>Bombus terrestris</i> CHCl ₃ /CH ₃ OH (1:1, v/v) Triacylglycerols. Unusual fatty acids with 24, 26, and 28 carbon atoms were found in triacylglycerols. (Cvacka <i>et al.</i> , 2008; Jiros <i>et al.</i> , 2011)	GC-MS Column: HP-5 ms (30m×0.25 mm, 0.25μm). Conditions: 40 °C (1 min), then 50 °C/min to 140 °C, then 3 °C/min to 320 °C (20 min). HPLC-MS Columns: Two stainless steel Nova-Pak C18 columns (300mm×3.9mm, 150mm×3.9mm, a particle size of 4 μm) connected in a series. Phase: acetonitrile (A) and 2-propanol (B). A linear gradient from 0 to 70% of B in 108 min (1.0 mL/min) was followed by a linear gradient to 100% B (150 min, 0.7 mL/min).
<i>Zophobas atratus</i> Dichloromethane Fatty acids, fatty acids methyl esters, fatty alcohols, sterols (Gołębowski <i>et al.</i> , 2014; Słocińska <i>et al.</i> , 2013)	GC-MS Column: HP-5 (30m×0.25 mm, 0.25μm). Conditions: From 80 (held for 10 min) to 320°C at 4°C/min, and then held isothermal for 20 min.

using GC-MS (Gołębiowski *et al.*, 2012; Pannkuk *et al.*, 2013a). Triacylglycerol fractions can be analyzed using the MALDI-TOF technique (Matrix Assisted Laser Desorption Ionization - Time of Flight) (Ayorinde *et al.*, 1999; Gidden *et al.*, 2007; Pannkuk *et al.*, 2013b). Figure 2 shows the different techniques used in the analysis of the fat body of insects and Table 2 contains data on the most frequently applied analytical techniques used in the analysis of the fat body of insects. Compounds in the fat body are mainly triacylglycerols, phospholipids, fatty acids, fatty acids methyl esters, fatty alcohols and sterols (Table 2). Free fatty acids, triacylglycerols and fatty acid esters (methyl-, ethyl-, decyl-, dodecyl- and tetradecyl-), alcohols, glycerol, and cholesterol (Słocińska *et al.*, 2012; Gołębiowski *et al.*, 2014) are examples of compounds which have been discovered using GC-MS in the analysis of the fat bodies of larvae and pupae *Z. atratus*. Large differences were noted in the case of the free fatty acid content of the larvae, where the acid content increased 24 h and 48 h after the Tenmo-AKH injection. Concentrations of free fatty acids detected in the fat body of larvae markedly increased under AKH treatment. On the other hand, the contents of the free fatty acids found in the fat body of pupae decreased after an injection of Tenmo-AKH (Gołębiowski *et al.*, 2014). The total amount of lipids identified in the pupae after using AKH was lower than the control (Słocińska *et al.*, 2012). In the case of lipids in larvae, an increase of cholesterol was observed 24 h after the introduction of the hormone, whereas after 48 h the amount of cholesterol decreased again (Gołębiowski *et al.* 2014).

Conclusions

The use of modern analytical techniques, specific and sensitive bioassays and molecular biology have rapidly accelerated the development of insect neuroendocrinology. The use of GC-MS allows both qualitative and quantitative determinations of the lipid composition of insects to be made, which means correlations can be observed between the state of development of insects and their energy demands as well as changes in the quantity and quality of lipids in the tested insect species.

The fat body plays a particular role in many of the processes related to the metabolism and life processes of insects. When there is a high demand for energy, for example during the flight of an insect, a continuous release of energy is necessary. Mobilization is governed primarily by carbohydrates and lipids, which are stored in the fat body (van der Horst, 1997). The mobilization of metabolites occurs when relevant hormones are released by the neuroendocrine system. In certain insect species, usually more than one type of hormone exists. For example, in the fat body of *L. migratoria*, there are three different forms of AKH (AKH-I, AKH-AKH-II and III) which among other things have different lengths of peptide chains. In his research, van der Horst drew attention to the influence of AKH on the amounts of energy, as well as the speed of its release. Even after 1 min, a 200 % increase in the

intracellular cAMP can be observed, compared to its initial level. Upon further lapses of time, only decreases in the amount of the cAMP are recorded and after 30 min, cAMP levels are already at the same output level of a signaling molecule. This shows that the effective length of time of the AKH action is very short (van der Horst, 1997). However, a series of biochemical changes can take place which cause changes in the metabolism of lipids and proteins, but whose effects can be seen only after longer periods of time (for example after 24 or 48 h). The length of time depends largely on the nature of the hormone, the species, the physiological conditions and the stage of development of the test insect.

In insects undergoing a complete transformation of the body, there is a total reorganization of the larvae, through the pupal stage right up to the final form of the insect (imago) (Larsen, 1976). So-called apoptosis - programmed cell death (PCD) - occurs in the tissue of the fat body. This process takes place at different phases of the development stage. For example, in the tobacco hornworm (*Manduca sexta*), PCD occurs during the 3 to 5 day larval stage (Müller *et al.*, 2004), and in the case of the silkworm (*Bombyx mori*) PCD occurs 2 days before pupate (Gui *et al.*, 2006, Lee *et al.*, 2009). The process of transformation during metamorphosis can also vary, tissue can differentiate between pupal and imago (Oberlander, 1985) and it may be subject to changes without moving the cells, or by moving the differentiation of larval cells again from primary cells (Kaneko, 2011). During metamorphosis, in the majority of insects, cells experience a period of larval and pupal transition (Larsen, 1976). In the course of intensive developmental changes, the lipid composition of the fat body also changes, especially free fatty acids and cholesterol (Słocińska *et al.*, 2012). Insects do not synthesize sterols and therefore sterols (primarily cholesterol) are essential components of their diet (Roller *et al.*, 2010). The very different impacts of AKH on the fat bodies of intensive feeding larvae and on the use of previously stored energy reserves in the beetle pupae *Z. atratus* (Gołębiowski *et al.*, 2014) may indicate the diverse roles this hormone plays in the indirect control of insect metabolism.

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