HORMONAL CONTROL OF FLIGHT METABOLISM IN ODONATA ?

MARKUS PETER-ERIK JANSSENS

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1.2 Abstract

Members of the AKH/RPCH family of peptides were identified in corpora cardiaca of the dragonfly *Anax imperator* (Anisoptera: Aeshnidae), *Orthetrum julia falsum* (Anisoptera: Libellulidae) and the damselflies *Pseudagrion inconspicuum* and *Ischnura senegalensis* (Zygoptera: Coenagrionidae). After isolation of the peptides by reversed phase high performance liquid chromatography, the primary structures were established by Edman sequencing and mass spectrometry (*Ani*-AKH: pGlu-Val-Asn-Phe-Ser-Pro-Ser-TrpNH$_2$), (*Lia*-AKH: pGlu-Val-Asn-Phe-Thr-Pro-Ser-TrpNH$_2$) and (*Psi*-AKH: pGlu-Val-Asn-Phe-Thr-Pro-Gly-TrpNH$_2$).

One corpus cardiacum of *A. imperator* contains about 40 pmol *Ani*-AKH, *O. julia* 19-24 pmol *Lia*-AKH and *P. inconspicuum* about 2.4 pmol *Psi*-AKH. Injection of *Ani*-AKH (3.4 pmol) increased the concentration of haemolymph lipids in *A. imperator*. *Lia*-AKH (1 pmol) similarly had an adipokinetic effect in *O. julia*. *Psi*-AKH (1 pmol) had an adipokinetic effect, as well as a small hyperglycaemic effect in *P. inconspicuum*.

The AKH peptides of other Odonata were investigated. In the suborder Anisoptera, *Ani*-AKH was identified in representatives of the Aeshnidae, Cordulegasteridae, and possibly the Corduliidae. *Lia*-AKH was identified in representatives of the Libellulidae and Gomphidae. In the suborder Zygoptera, *Psi*-AKH was identified in representatives of the families Chlorolestidae, Lestidae and Chlorocyphidae, and possibly the Calopterygidae and Protoneuridae.

Classification of Odonata according to their flight behaviour as "perchers" or "fliers" is supported by parameters of energy metabolism. Lipid metabolism seems to have a greater importance in fliers than perchers. The lipid concentration in the haemolymph is highest in the flier *A. imperator*, intermediate in the percher *O. julia* and lowest in the percher *P. inconspicuum*. There are indications that mitochondria isolated from flight muscles of *A. imperator* may have a higher capacity for lipid oxidation than *O. julia*. The contribution of carbohydrates to flight metabolism seems to be more important in perchers than in fliers. The concentration of carbohydrates in the haemolymph is highest in *P. inconspicuum*, intermediate in *O. julia* and lowest in *A. imperator*. The maximal activity of phosphofructokinase (a rate-limiting enzyme of glycolysis) is higher in the percher, *O. julia*, than in the flier, *A. imperator*.

The lipid concentration in the haemolymph is higher than that of the carbohydrates in *O. julia*, *A. imperator* and *P. inconspicuum*. Palmitoyl-carnitine is oxidised at high rates by isolated mitochondria from flight muscles of *O. julia* and *A. imperator*, similar to *Locusta migratoria*. Lipid is the major fuel utilised during flight in *O. julia*. Carbohydrates (in the haemolymph) and proline (in the haemolymph and flight muscles) are utilised as minor fuels.

It is concluded that the processes of lipid metabolism provide the major source of energy during flight in Odonata. The AKH peptides seem to play a role in regulating lipid mobilisation during flight in Odonata.

1.3 Abbreviations

Amino acids:

A, Ala	alanine
D, Asp	aspartate
G, Gly	glycine
I, Ile	isoleucine
L, Leu	leucine
N, Asn	asparagine
F, Phe	phenylalanine
P, Pro	proline
S, Ser	serine
T, Thr	threonine
W, Trp	tryptophan
Y, Tyr	tyrosine
V, Val	valine

Other abbreviations:

ACN	acetonitrile
ADP	adenosine-5'-diphosphate
AKH	adipokinetic hormone
AMP	adenosine-5'-monophosphate
Ani-AKH	*Anax imperator* adipokinetic hormone
ATP	adenosine-5'-triphosphate
AUFS	absorbance units full scale
Bld-HrTH	*Blaberus discoidalis* hypertrehalosaemic hormone
CC	corpus cardiacum; corpora cardiaca
CoA	coenzyme A
CPM	counts per minute
CS	citrate synthase, EC 4.1.3.7
Cytox	cytochrome oxidase, EC 1.9.3.1
DPM	disintegrations per minute
DTE	dithioerythritol

DTNB	5,5'-dithio-bis(2-nitrobenzoic acid)
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
FABMS	fast atom bombardment mass spectrometry
GAPDH	glyceraldehyde-3-phosphate dehydrogenase, EC 1.2.1.12
Grb-AKH	*Gryllus bimaculatus* adipokinetic hormone
HOAD	3-hydroxyacyl-CoA dehydrogenase, EC 1.1.1.35
HrTH	hypertrehalosaemic hormone
LDH	lactate dehydrogenase
Lia-AKH	*Libellula auripennis* adipokinetic hormone
Lom-AKH-I	*Locusta migratoria* adipokinetic hormone I
Mem-CC	*Melolontha melolontha* corpus cardiacum peptide
NAD^+	nicotinamide adenine dinucleotide
NADH	nicotinamide adenine dinucleotide (reduced)
$NADP^+$	nicotinamide adenine dinucleotide phosphate
NADPH	nicotinamide adenine dinucleotide phosphate (reduced)
Pab-RPCH	*Pandalus borealis* red pigment-concentrating hormone
PCA	perchloric acid
Pea-CAH-I	*Periplaneta americana* cardioacceleratory hormone I
PFK	phosphofructokinase, EC 2.7.1.11
PK	pyruvate kinase, EC 2.7.1.40
Poa-HrTH	*Polyphaga aegyptiaca* hypertrehalosaemic hormone
Psi-AKH	*Pseudagrion inconspicuum* adipokinetic hormone
PTH	phenylthiohydantoin
RP-HPLC	reversed phase high performance liquid chromatography
TFA	trifluoracetic acid
TMPD	N, N, N', N'-tetramethyl-p-phenylenediamine
TRA	triethanolamine
U	enzyme units (µmol product formed/min)

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2. INTRODUCTION

Peptidic regulators from the neuroendocrine system control many physiological processes in insects. These processes include moulting, regulation of water balance, contraction of the hindgut, and energy metabolism (Gäde, 1992a). Flight is energetically the most demanding activity of insects. To power flight a continuous supply of substrates to the flight muscles is essential. If sufficient quantities of fuel are not immediately available, they must be mobilised from elsewhere. Support for the hypothesis that insects possess a hormone that controls the mobilisation of substrates was provided by studies using cockroaches and locusts. The cockroach, *Periplaneta americana* and locusts, *Locusta migratoria* and *Schistocerca gregaria*, were injected with an extract prepared from their corpora cardiaca (CC). The concentration of carbohydrates and lipids in the haemolymph of the cockroach and locusts, respectively, were found to increase dramatically as a consequence of this injection (Steele, 1961; Beenakkers, 1969b; Mayer and Candy, 1969). The factor in the CC of the locust responsible for this adipokinetic effect was fully characterised by Stone *et al.* (1976). The compound, a peptide now named *Lom*-AKH-I according to the nomenclature of Raina and Gäde (1988), has many structural similarities to a previously characterised chromatophorotropin, called *Pab*-RPCH, an octapeptide isolated from the neurosecretory X-organ/sinus gland complex in the eyestalk of the Crustacean, *Pandalus borealis* (Fernlund and Josefsson, 1972). A new family of neuropeptides was discovered, and named the adipokinetic hormone/red pigment-concentrating hormone (AKH/RPCH) family, based on the functions of the first members fully characterised.

Improved technologies enabling structural determination of minute quantities of these peptides, tremendously accelerated the discovery of new neuropeptides, including peptides of the AKH/RPCH family. To date at least 24 different bioanalogues have been discovered in insects (Gäde *et al.*, 1994).

2.1 Structure of AKH/RPCH peptides

The peptides of the AKH/RPCH family are octa-, nona-, or decapeptides and have N- and C-blocked termini. The absence of free N-terminal amino and C-terminal carboxyl groups contributes to their biostability by making the peptides more resistant

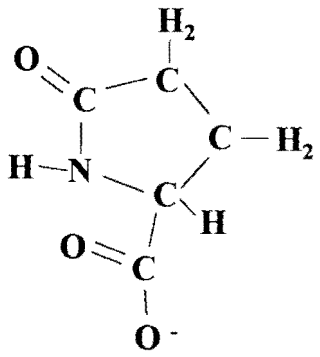


Fig. 1. Structure of pyroglutamate

to degradation by exopeptidases. N-blocked termini are a feature of other peptide families such as the RFamides in the Coelenterates (Grimmelikhuijzen *et al.*, 1994). The blocked termini include pyroglutamate (5-oxopyrrolidine-2-carboxylic acid; pGlu) (Fig. 1), which is formed by enzymatic cyclisation of an N-terminal Gln residue (Pohl *et al.*, 1991). All the AKH/RPCH peptides discovered to date have an N-terminal pGlu residue, whereas the RFamides contain other N-terminal modifications such as a L-3-phenyllactyl group.

The C-terminal blocking involves an amidation of the free carboxyl group, and is a characteristic feature of many peptide hormones. The nitrogen atom of the amide group is derived from the nitrogen of a C-terminal Gly in the prohormones of peptides such as oxytocin, gastrin and α-MSH (Bradbury and Smyth, 1991). The C-terminal modification in the AKH/RPCH peptides very likely occurs by the mechanism of Bradbury *et al.* (1982). Evidence for this type of modification in octapeptides of the AKH/RPCH family of peptides is provided by the fact that the precursor molecule of the octapeptide *Scg*-AKH-II contains a Gly residue at the required position (O'Shea and Rayne, 1992). Gly is also present at position 9 of all the nona- and decapeptide forms of the AKH/RPCH family (Gäde *et al.*, 1994) indicating that there may be a Gly in the required position in the prohormones of the octapeptides of the AKH/RPCH peptides.

Other common features of peptides of the AKH/RPCH family are the presence of aromatic amino acids at positions 4 and 8, and all but two members are not charged. The amino acid substitutions observed at each position are indicated in Table I.

Table I. Amino acid substitutions of the AKH/RPCH family

1	2	3	4	5	6	7	8	9	10	
pGlu	Val	Asn	Phe	Ser	Pro	Asn	Trp	Gly	Asn	NH_2
	Leu	Thr	Tyr	Thr	Thr	Gly			Thr	
	Ile				Ser	Ser			Tyr	
					Ala	Asp				
						Trp				

2.2 Distribution of AKH/RPCH peptides

Most AKH/RPCH peptides have been sequenced from the orders Orthoptera, Phasmida, Dictyoptera, Coleoptera, Diptera and Lepidoptera (Gäde *et al.*, 1994). An adipokinetic peptide was sequenced from the dragonfly *Libellula auripennis* (Anisoptera: Libellulidae). This peptide was named *Lia*-AKH as it had adipokinetic activity when conspecifically injected (Gäde, 1990a).

The Odonata is the most primitive insect order investigated to date for their AKH peptides (Gäde *et al.*, 1994). The amino acid sequence of *Lia*-AKH seemed consistent with the requirements for the structure of a putative ancestral insect AKH peptide (Gäde, 1990a). However, the Libellulidae is the most advanced family within the Odonata (Fraser, 1957; Trueman, 1991). Preliminary results also indicated the presence of another adipokinetic compound in CC of the emperor dragonfly, *Anax imperator* (Anisoptera: Aeshnidae) (Gäde, pers. comm.). The presence of an adipokinetic compound with the same retention time on RP-HPLC as *Lia*-AKH was also demonstrated in CC of a dragonfly of the genus *Aphylla* (Anisoptera: Gomphidae) (Gäde, pers. comm.). It was therefore of interest to extend the available structural data of AKH peptides to other families of the Odonata, and to comment on the identity of the ancestral AKH/RPCH peptide.

2.3 Odonata phylogeny

In southern Africa, the Odonata are represented by two suborders: the Anisoptera (dragonflies) and Zygoptera (damselflies). No representatives of the third extant suborder, the Anisozygoptera occur (Pinhey, 1951). It has generally been accepted, based only on morphological characters, that the dragonfly ancestors were members of

the Zygoptera. This suborder includes the family Coenagrionidae, representing the most primitive extant Odonata (Fraser, 1957; Trueman, 1991). Recently, however, it was proposed that the Zygoptera are a monophyletic entity, and the ancestral odonates belonged to the Anisozygoptera, based on a study of the ground plan of odonate wing venation and the microsculpture of the pterostigma (Bechly, 1993). No phylogeny of the Odonata that has been published to date derives any of its conclusions from molecular information. The amino acid sequences of the AKH/RPCH peptides are highly conserved, and different peptides occur in a family-specific manner (Gäde *et al.*, 1994). The structural data contained in the AKH peptides of the Odonata could therefore be potentially useful in providing an independent measure of phylogenetic relationships of the families and/or suborders of the Odonata.

2.4 Function of AKH/RPCH peptides

The AKH/RPCH family peptides are structurally related, but functionally very diverse. Physiological effects ascribed to AKH/RPCH peptides include hyperlipaemia, which led to their initial discovery as regulators of the supply of metabolites to support energy demanding activities (for a review of some functions of AKH/RPCH peptides see Gäde, 1992b). The adipokinetic hormone of locusts has been shown to mobilise diglycerides from the fat body, into the haemolymph. The peptides are thought to act on the fat body via adenylate cyclase, increasing fat body cAMP levels (for a review of some functions of AKH/RPCH family peptides, see Goldsworthy and Mordue, 1989). The haemolymph proteins and carrier lipoproteins of the locust are regrouped, increasing the lipid-carrying capacity of the haemolymph (Goldsworthy and Mordue, 1989). A continuous supply of the fatty acid moieties is thus available in the flight muscle for oxidation to supply the required energy during flight. The adipokinetic hormone also stimulates lipid oxidation over carbohydrate metabolism in the flight muscles of locusts (Goldsworthy, 1983). The peptides play a role in carbohydrate metabolism, causing hypertrehalosaemia (Gäde, 1992a), activation of glycogen phosphorylase in the fat body of locusts, cockroaches (van Marrewijk *et al.*, 1983) and tobacco hornworm larvae, *Manduca sexta* (Siegert and Ziegler, 1983). Effects of the peptides in the inhibition of protein biosynthesis by the fat body (Carlisle and

Loughton, 1979) and inhibition of vitellogenesis have been described (Carlisle and Loughton, 1986). Other functions of the AKH/RPCH peptides include suppression of fatty acid synthesis, developmental regulation of haeme synthesis for mitochondrial cytochromes, induction of gene expression for a cytochrome P450 enzyme, and enhanced competency for fat body protein biosynthesis (Keeley *et al.*, 1994). Neurohormone D, also known as *Pea*-CAH-I, increases the amplitude and frequency of heartbeat in semi-isolated cockroach heart preparations (Baumann *et al.*, 1990). The adipokinetic hormone may also be involved during reproduction, as it was established that the titre of adipokinetic hormone in the haemolymph increases at the end of the ovarial cycle (Moshitzky and Applebaum, 1990). The mobilisation and transport of lipids to the oocytes could therefore be controlled by this hormone (for a discussion of this possible function, see Gäde, 1992a).

Depending on the metabolic specialisation of a particular insect, the AKH/RPCH peptides can mobilise different energy substrates. Injection of *L. migratoria*, with extracts containing AKH/RPCH peptides results in an adipokinetic effect (the concentration of lipids in the haemolymph increases). In *P. americana*, there is a pronounced hypertrehalosaemic effect when injected with the same substances. The qualitative difference in this response is not dependent on which peptide is injected, but on the difference in the cell-mediated response in the fat body after binding of the hormone to its receptor. The purification of compounds such as neuropeptides is greatly simplified if a reliable, rapid, and inexpensive test to demonstrate their presence is available. The adipokinetic effect in locusts and hyperglycaemic effect in cockroaches provides biotests to screen a large number of samples for the presence of putative adipokinetic/hypertrehalosaemic compounds (Gäde, 1990b).

Apart from screening fractions for desired compounds, an indication of the possible physiological role of putative adipokinetic/hypertrehalosaemic compounds can be established by performing a bioassay in the species under investigation. An adipokinetic, and a small hyperglycaemic effect was demonstrated as a consequence of conspecific injection of an extract from CC, as well as injection of synthetic *Lia*-AKH into the dragonfly *L. auripennis* (Gäde, 1990a). An adipokinetic effect after injection of conspecific CC extract had also been reported in larvae of the dragonfly *Tramea*

virginia (Anisoptera: Libellulidae) (Tembhare and Andrew, 1991). One aim of this project therefore was to provide a basis for studies into the postulated role of the dragonfly adipokinetic peptides in regulating energy metabolism.

2.5 Metabolic fuels

The energy requirements of tissues such as flight muscle are provided by the hydrolysis of ATP. ATP is resynthesised in the processes of fuel oxidation. It is well established that different fuels are used by different muscles, and at different stages of development. The qualitative differences in fuel utilisation depend on the physiological role of the muscle, and reflect an adaptation of this tissue to meet the requirements of the animal (Crabtree and Newsholme, 1975). Many different methods have been used to estimate which fuel is oxidised by muscles. Direct methods include measurements, during flight of insects, of the ratio of CO_2 production to O_2 consumption (respiratory quotient), and of changes in the content of different substrates in the haemolymph and tissues (Jutsum and Goldsworthy, 1976).

Indirect methods such as measurement of the maximal activities of key (non-equilibrium) enzymes of carbohydrate and lipid breakdown, have been used to categorise insects according to the specialisation of their flight muscles in fuel utilisation. An enzyme catalysing a non-equilibrium reaction is responsible for the flux-generating step of a metabolic pathway (Crabtree and Newsholme, 1975; Newsholme and Crabtree, 1986). By comparing the ratios of enzymes belonging to different pathways (the so-called constant proportion approach), information about their quantitative significance for metabolism can be obtained, and make it possible to discern distinct metabolic types of muscle. (Pette, 1965; Bass *et al.*, 1969; Beenakkers, 1969a; Beenakkers *et al.*, 1975). The enzymes used by the authors of this second approach were not necessarily non-equilibrium enzymes, but they still allowed for broad conclusions to be drawn, especially when comparing the change in the activity of such enzymes during the insect's life cycle.

Data from the rate of oxidation of different substrates by mitochondrial preparations from the flight muscle have been successfully used to elucidate the likely metabolic

pathways of energy metabolism by measuring which substrates are preferentially catabolised in insects such as the tsetse fly, *Glossina morsitans* (Bursell and Slack, 1976) and the Colorado potato beetle, *Leptinotarsa decemlineata* (de Kort *et al.*, 1973).

Long-term locomotor activities in many different organisms are powered by lipids as the major fuel, since the energy yield per unit mass of lipid, compared with that of substrates such as carbohydrates is much higher: 129 mol ATP is produced from the complete oxidation of 1 mol palmitate (260.5 g), thus yielding 0.5 mol ATP/g, whereas in the case of 1 mol glucose (180.2 g), 36 mol ATP is produced from its complete oxidation, yielding 0.2 mol ATP/g.

Lepidoptera that do not feed during the adult stage utilise primarily lipids as energy source and have to rely exclusively on food reserves derived from larval feeding. Other Lepidoptera that feed on nectar may utilise carbohydrates as well as lipids (Beenakkers, 1969a). Locusts use a combination of lipids and carbohydrates: in the initial phase of flight mainly carbohydrates are used as these are readily available in high concentration in the haemolymph. During the first 30 min of flight the rate of lipid oxidation in the flight muscles increases concomitantly with a rising lipid concentration in the haemolymph. The lipid concentration in the haemolymph increases as a result of being mobilised by the adipokinetic hormone from stores in the fat body (Goldsworthy, 1990). Many Diptera and Hymenoptera use carbohydrates exclusively as energy substrate (Sacktor, 1965).

Odonata are some of the best fliers in the insect world, both in terms of speed, and in the long migrations some of them are known to undertake. The dragonfly, *Pantala flavescens*, has a world-wide distribution, and is known as an active flier. There have been reports of large numbers of *P. flavescens* arriving at a ship which was 466 km from the nearest land (Corbet, 1962). Indications that lipids play a major role in supporting flight in *P. flavescens* were experimentally supported by the apparent ability of flight muscle homogenates and mitochondrial preparations to oxidise fatty acids (Kallapur and George, 1973). In another study, a highly significant reduction of

diglycerides in *P. flavescens* flight muscle after periods of flight was described (Kallapur *et al.*, 1979).

The content of Pro in tissues and haemolymph has been shown to decrease after flight, in a variety of insects such as *G. morsitans* (Bursell, 1975), *L. decemlineata* (Weeda, 1981), in the African fruit beetle, *Pachnoda sinuata* (Zebe and Gäde, 1993), in the blister beetle, *Decapotoma lunata* (Auerswald and Gäde, 1995) and even in the locust, *L. migratoria* (Worms and Beenakkers, 1980). Bursell (1981) suggested that Pro is partially oxidised according to the reaction:

$$Pro + 2.5\ O_2 + 14\ ADP \rightarrow Ala + H_2O + 2\ CO_2 + 14\ ATP$$

Bursell (1981) reached this conclusion as the initial depletion of Pro in *G. morsitans* was associated with a roughly stoichiometric increase in Ala. Proline is proposed to be resynthesised from the accumulated Ala. A high energy yield per unit weight for the part of Pro that is oxidised was calculated (0.52 mol ATP/g) similar to the energy yield obtained from oxidation of lipid. In some insects, such as the Japanese beetle, *Popillio japonica*, the proportion of Ala recovered is much lower, indicating that a higher proportion of the Pro is subjected to complete, rather than partial oxidation (Hansford and Johnson, 1975). Bursell (1981) calculated that the energy yield for the complete oxidation of Pro is 0.3 mol ATP/g.

In the dragonfly, *Mesogomphus lineatus* (Anisoptera: Gomphidae), a reduction of the concentration of Pro in the haemolymph after periods of flight was reported (Subramanian and Varadaraj, 1985). Proline could therefore also play a possible role in supporting flight in dragonflies.

2.6 Flight behaviour of Odonata: "Perchers" and "Fliers"

Dragonflies were classified by Corbet (1962) according to thermoregulatory properties, to either belong to the typical "fliers" which when active remain constantly on the wing or "perchers" which only make short, trivial flights and then return to a perch. This distinction has also been used to explain differences in the strategies Odonata employ to catch prey (Corbet, 1980). Members of the Aeshnidae and

Corduliidae are typically fliers, while most perchers belong to the Zygoptera, Gomphidae and Libellulidae (Corbet, 1962).

The distinction between fliers and perchers has been studied extensively, especially with regard to thermoregulation. Generally, a certain thoracic temperature has to be attained by a dragonfly, before it will spontaneously take off. Fliers increase thoracic temperature by "wing whirring" (Moore, 1953) and, in flight, thermoregulate exclusively via a control of blood circulation, whereas perchers gain heat passively, by behavioural adjustments (May, 1978; Heinrich and Casey, 1978). The ambient temperature was found to influence take-off in fliers less than perchers (Vogt and Heinrich, 1983). The differences between perchers and fliers was shown to extend to differences in morphological characteristics, such as wing length and thoracic diameter, volume, and mass (May, 1981). In addition there is some evidence that fliers have significantly higher resting metabolic rates at a given body mass than perchers (May, 1979).

Two species of dragonfly that are common to Cape Town belong to each of these categories. *Orthetrum julia* Kirby *falsum* Longfield (Anisoptera: Libellulidae) is a typical percher. *Anax imperator* Leach *mauricianus* Rambur (Anisoptera: Aeshnidae) was observed to be a flier (Moore, 1953). It was of interest to examine these two species for any correlation between parameters of energy metabolism and flight behaviour.

3. MATERIALS AND METHODS

3.1 Statistical methods

Student's *t*-test has been employed where the significance of the difference between two independent groups was determined. Student's paired *t*-test was used where treatments could be compared pair-wise. It is invalid to employ multiple *t*-tests to examine the differences between all possible pairs of means. Multiple comparisons between means were performed using the Tukey test, after significant differences between at least one pair had been established using analysis of variance (ANOVA). All statistical tests were according to Zar (1984).

3.2 Experimental animals

Permission to perform animal experiments was obtained from the Animal Ethics Committee, University of Cape Town. Insects were sacrificed after anaesthetising by keeping them at 4°C.

3.2.1 *Locusta migratoria*

Adult male migratory locusts, *L. migratoria,* from our own colony were used. The locusts were held under crowded conditions at a temperature of 32 ± 2°C, a relative humidity of 30%, with a light : dark 14:10 h cycle. They were fed with fresh grass twice daily, supplemented with rolled oats. The locusts used in experiments were of similar physiological age, and used 15 to 25 days after the final moult.

3.2.2 *Periplaneta americana*

Adult male cockroaches, *P. americana*, were obtained from our own colony which was maintained under crowded conditions at a temperature of 25°C, a relative humidity of 50%, with a light : dark 14:10 h cycle. They were fed with a mixture of dog food and Pronutro, and provided with water *ad libitum*.

Table II. Odonata collected during the study

	Location	Date
Suborder: Zygoptera		
Family: Chlorolestidae		
Chlorolestes fasciata	Giant's Castle Game Reserve[a]	Jan. 1993
Chlorolestes tessellata	Palmiet River, Grabouw[b]	Feb. 1992
Family: Lestidae		
Lestes tridens	Queen Elizabeth Park Nature Reserve[c]	Jan. 1993
Lestes plagiatus	Giant's Castle Game Reserve[a]; Hilton, PMB[a]	Jan. 1993
Family: Calopterygidae		
Phaon iridipennis	Sabie River, KNP[c]	Jan. 1993
Family: Chlorocyphidae		
Chlorocypha caligata	Sabie River, KNP[c]	Jan. 1993
Family: Protoneuridae		
Allocnemis leucosticta	Hilton, PMB[a]	Jan. 1993
Family: Coenagrionidae		
Pseudagrion inconspicuum	See text for details	Mar. 1993-'94
Ischnura senegalensis	See text for details	Mar. 1993-'94
Suborder: Anisoptera		
Family: Gomphidae		
Ictinogomphus ferox	Skukuza, KNP[c]	Jan. 1993
Ceratogomphus pictus	Hilton, PMB[a];	Jan. 1993
	Nossob, KGP[d]	Jan. 1994
Family: Cordulegasteridae		
Anotogaster sieboldii	Tsukuba, Japan	Sept. 1992; '93
Family: Aeshnidae		
Aeshna miniscula	Palmiet River, Grabouw[b]; Cathedral Peak Nature Reserve[a]	Feb. 1992; Jan. 1993
Aeshna subpupillata	Giant's Castle Game Reserve[a]	Jan. 1993
Anax imperator mauricianus	See text for details	Feb. 1992-'94
Anax speratus	Palmiet River, Grabouw[b]; Giant's Castle Game Reserve[a]	Feb. 1992
Family: Corduliidae		
Syncordulia gracilis	Palmiet River, Grabouw[b]	Feb. 1993
Family: Libellulidae		
Orthetrum julia falsum	See text for details	Feb. 1992-'94
Crocothemis erythraea	Maynardville Park, CT[b]	Feb. 1992
Bradinopyga cornuta	Sabie River, KNP[c]	Feb. 1993
Sympetrum vicinum	Amherst, U.S.A.	
Sympetrum infuscatum	Tsukuba, Japan	
Trithemis dorsalis	Hilton; Botanical Gardens, PMB[a]	Jan. 1992
Trithemis stictica	Hilton; Botanical Gardens, PMB[a]	Jan. 1992
Trithemis arteriosa	UCT dam, CT[b]	Feb. 1993
Brachythemis leucosticta	Umhlanga[a]; Skukuza, KNP[c]	Jan. 1993
Pantala flavescens	Skukuza, KNP[c]	Jan. 1993

Odonata were caught in the provinces of (a) KwaZulu-Natal; (b) Western Cape; (c) Eastern Transvaal; and (d) Northern Cape. Abbreviations: Kruger National Park (KNP), Kalahari Gemsbok National Park (KGP), Pietermaritzburg (PMB), University of Cape Town (UCT), Cape Town (CT).

3.2.3 Odonata

Anisoptera (dragonflies) were collected over a period of two years spanning the austral summers of 1991/1992, 1992/1993 and 1993/1994. Zygoptera (damselflies) were collected during the last two seasons. All the individuals were of unknown age.

Orthetrum julia falsum is very common in Cape Town. It was therefore ideally suited to determine whether the CC of this dragonfly also contain *Lia*-AKH, similar to the adipokinetic octapeptide that had been isolated earlier from CC of *L. auripennis*, belonging to the same family (Gäde, 1990a). *A. imperator* was the only dragonfly, belonging to a family other than the Libellulidae, that was relatively abundant locally. This species was interesting because the Aeshnidae are more primitive than the Libellulidae (Fraser, 1957; Resh and Solem, 1984). The most primitive extant Odonata however, are damselflies (Zygoptera) belonging to the family Coenagrionidae (Fraser, 1957). Two members of this family, *Pseudagrion inconspicuum* Ris and *Ischnura senegalensis* Rambur, were abundant in Cape Town.

Adult dragonflies of the species *O. julia* and *A. imperator* were caught by netting from the wild in Cape Town. *A. imperator* was observed to be most abundant along natural banks of the Liesbeeck River (Mowbray), the banks of the Rietvlei wetlands (Milnerton), on the main pond in Maynardville Park (Wynberg), and on the ponds along the river running through Wynberg Park (Wynberg). *O. julia* was much more abundant than *A. imperator*, and collected in Wynberg Park (Wynberg), the canalised tributary of the Black River in Keurboom Park (Claremont), as well as non-canalised banks of the Liesbeeck River (Mowbray), along canalised parts of the river flowing into Maynardville Park (Wynberg) and on the banks of the dam on the Main Campus of the University of Cape Town. The dragonflies were kept on ice in envelopes to prevent wing flapping and to keep their metabolic rate low, and transferred to the laboratory as soon as possible after capture. When used for the determination of enzyme activities, or the isolation of mitochondria from the flight muscles, the dragonflies were kept at 4°C at high humidity for up to three days. This was considered acceptable, as dragonflies used in a study to determine electrophysiological

aspects of flight behaviour had been kept refrigerated for up to 5 days before use (Pond, 1973).

Adult specimens of *P. inconspicuum* and *I. senegalensis* were collected in the wild by netting in Cape Town on the banks of the Liesbeeck River, Mowbray. After capture, pairs of damselflies were immediately placed into 40 ml closed containers filled with moist paper towel, and kept on ice to prevent wing movement and evaporative water loss. The damselflies quietened down as soon as they gripped onto the paper tissue. They were transferred to the laboratory within 2 h of capture.

Other dragon- and damselfly species for isolation of peptide material were caught, kept on ice in envelopes, and transferred to the laboratory as soon as possible. They were representatives of the families Cordulegasteridae, Aeshnidae, Libellulidae, Gomphidae, Corduliidae, Coenagrionidae, Chlorolestidae, Lestidae, Calopterygidae, Chlorocyphidae and Protoneuridae. These dragonflies were caught during collecting trips to KwaZulu-Natal (January 1992, 1993), the Kruger National Park, Eastern Transvaal (January 1993), as well as the Kalahari Gemsbok National Park, Northern Cape (January 1994) and are listed in Table II.

3.3 The corpora cardiaca

The CC, the major neurohaemal organ of insects, are mostly located in the head behind the brain, but are sometimes found in the neck or anterior part of the prothoracic segment. They are paired, and are often merged with the lateroventral walls of the aorta. The CC are connected to neurosecretory centres in the brain by the nervi corporis cardiaci. The CC also contain glandular (intrinsic) cells, which are sometimes separated from the neurosecretory axons coming from the brain (Raabe, 1989). The CC of Odonata are paired structures which form an annular thickening around the anterior part of the aorta. The neurosecretory axons (extrinsic) and glandular (intrinsic) cells are not arranged in separate zones (Schaller and Charlet, 1983). Neurosecretory granules were observed in intrinsic and extrinsic cells of the CC of *Aeshna cyanea* (Tembhare, 1980). The CC have been found to be the best source of extraction of neuropeptides of the AKH/RPCH family (Gäde, 1990b).

3.3.1 Preparation of extracts from CC

Dragonflies and damselflies were sacrificed by making a vertical section between the pro- and mesothorax. A sagittal section of the head was made just above the level of the mid-eye. This preparation was secured in a wax-filled dish, with pins through the thorax and one eye. The blueish-white CC were then clearly visible, and could be removed, often attached to the aorta, with watchmaker's forceps, and deposited into 200 µl 80% methanol in plastic 1.5 ml tubes.

The CC were disrupted in the cold using an ultrasonic homogeniser (Branson) (2 x 10 s.) and then centrifuged for 3 min at 10 000 g using a Biofuge 15 centrifuge (Heraeus). The pellet was extracted with a second quantity of 200 µl 80% methanol, centrifuged, the supernatants combined, and dried down in a vacuum centrifuge (Bachofer).

3.4 Identification of bioactive compounds

3.4.1 Bioassays in *L. migratoria* and *P. americana*

Adult, male locusts were kept under funnels, and adult, male cockroaches in small containers for at least 2 h at room temperature before the start of the experiment. A 1 µl haemolymph sample was collected just before the animal was injected with the extract, compound or chromatographic fraction to be analysed, in a volume of 10 µl H_2O. A second haemolymph sample was taken 90 min (locusts) or 120 min (cockroaches) later and the haemolymph lipids of the locusts (measured as vanillin-positive substances using cholesterol as standard) or the haemolymph carbohydrates of the cockroaches (measured as anthrone-positive compounds using glucose standards) determined as described below.

3.4.2 Bioassays in Odonata

Adult dragonflies of the species *A. imperator* (both sexes) and *O. julia* (males) were used for bioassays. After collecting as described previously, the dragonflies were kept at 10°C for 6 h. Their wings were restrained with modelling clay, the dragonflies were provided with a piece of plastic tubing onto which they could cling, and funnels were

placed over them. To prevent dehydration, a piece of cotton wool soaked in water was placed in front of their mouthparts, and they invariably gripped onto this with their mandibles. Haemolymph samples of either 0.5 or 1 µl were taken from the dorsal side of the thorax, between the wing attachments and collected into 200 µl H_2SO_4. The dragonflies were then injected with a solution of 1 µl into the abdomen. A second haemolymph sample was obtained 90 min later. The test tubes containing the haemolymph samples were sealed and allowed to warm to room temperature. After mixing well, 100 µl were pipetted into a second test tube, and the lipids and carbohydrates determined in alternate aliquots.

Larvae of *A. imperator*, and a mixture of larvae of *O. julia* and *Crocothemis erythraea* (Anisoptera: Libellulidae) were collected by netting from submerged vegetation in flowing parts of a small river in Wynberg Park, Cape Town. They were transported to the laboratory within 2 h, placed into separate glass jars (volume ca. 1 l) and provided with a stick to which they could cling. They were left overnight in the dark at room temperature (20 to 25°C). Bioassays were performed in the morning, in a similar way as described previously for the adults, except that the larvae were dried carefully with paper towel before haemolymph sampling and injection, and then returned to the appropriate jar, until a second sample was taken 90 min later. In some cases the injection wound was sealed with wax. The jars were covered by black plastic bags during the course of the experiment.

Adult *P. inconspicuum* of both sexes were collected from the wild as described previously. They were left at room temperature for 2 h in the dark, prior to the experiment, in the containers they had been collected into. The damselflies were injected into the abdomen in a volume of 1 µl, and 0.5 µl haemolymph samples were taken 90 min later.

3.5 Colorimetric assays

3.5.1 Determination of total lipids

Vanillin reagent: Vanillin (1.98 g) was dissolved in 668 ml H_3PO_4 (concentrated) by constant stirring in the dark at 60°C. After the vanillin was completely dissolved, the solution was cooled and 332 ml H_2O added. The reagent was stored in the dark.

The sample to be analysed was introduced into 100 µl H_2SO_4 (concentrated) in a test tube, mixed thoroughly, heated to 100°C for 10 min and then cooled to room temperature in a water bath. 1 ml vanillin was added, mixed well, and incubated in the dark for 30 min. Reagent blanks contained no sample, but were otherwise treated in an identical way. The absorbance at 546 nm was measured using a photometer (Vitatron).

A standard curve was constructed using a standard solution of 0.5 mg/ml cholesterol in chloroform. Volumes of 20, 40, 60, 80, 120 and 160 µl of the cholesterol solution were pipetted into test tubes (each amount in triplicate). Reagent blanks contained 200 µl chloroform. The test tubes were heated to 100°C until all the chloroform was evaporated, and cooled to room temperature. At this stage the test tubes could be sealed with parafilm and stored at -20°C until needed. After 100 µl H_2SO_4 was added and the tubes well mixed, the determination continued as described above. The determination was according to Zöllner and Kirsch (1962) with minor adaptations.

3.5.2 Determination of total carbohydrates

Anthrone reagent: Anthrone (1.2 g) was added to 600 ml H_2SO_4 and stirred until dissolved. Water (300 ml) was then added dropwise (using a burette) to the cold solution (kept on an ice bath) with continuous stirring. The anthrone reagent was stored in the dark.

The sample to be analysed was introduced into 100 µl H_2SO_4, 2 ml anthrone reagent was added, mixed well, and heated to 100°C for 8 min. The mixture was cooled to room temperature and incubated in the dark for 30 min. The reagent blank

contained no haemolymph sample, but was otherwise treated exactly the same. The absorbance measured at 585 nm using a photometer (Vitatron).

A standard curve was constructed using glucose standards. A stock solution of 100 mg/ml glucose was diluted in a series to produce concentrations of 0.625, 1.25, 2.5, 5, 10, 15, 20, 25, 30, 40, and 50 mg glucose/ml H_2O. 1 µl of each dilution was introduced into 100 µl H_2SO_4, and the determination continued as described above. The determination was according to Spik and Montreuil (1964) with minor adaptations.

3.6 Isolation of CC peptides by RP-HPLC

Isolation of adipokinetic compounds from crude methanolic extracts of CC was achieved using reversed phase high performance liquid chromatography (RP-HPLC) as the separating technique (Gäde, 1985). The columns used included:
Nucleosil 100 C18, 4.6 mm x 125 mm, particle size 5 µm, equipped with a 20 mm guard column of the same material (Macherey and Nagel);
Waters Nova-Pak 3.9 x 150 mm, particle size 4 µm, pore size 60 Å: column supports were C18, C8 and phenyl.

Two HPLC systems were used, one system (Gilson) consisted of two model 302 piston pumps with a 5 S pump head, a manometric module Model 802, a Model 811 mixing chamber, a Rheodyne Model 7125 sample injector with a 50 µl sample loop, and an LKB 2151 variable wavelength detector (10 µl HPLC flow cell; 10 mm path length). A microcomputer (Apple II+) was used to control the pumps.

The second HPLC system (Beckman) was a System Gold equipped with Solvent Module 126, Detector Module 166, a Module 507 autosampler, a Module 406 analog interface and an IBM-compatible Samsung Computer SD 620. Peak areas could be integrated using the Beckman System Gold software for purposes of quantification.

The fluorescence monitor used was a model RF-535 (Shimadzu). Fluorescence was measured at an excitation wavelength of 276 nm, and an emission wavelength of 350 nm, to detect the presence of Trp. Fluorescence is expressed as µvolt output from

the detector, relative to 10 mV full scale output with the range selector on "1024" (the least sensitive setting).

Solvent A was 0.11% TFA and the composition of solvent B was 60% ACN and 0.1% TFA. Using the Nucleosil C18 column, separation was achieved using a linear gradient from 43 to 53% solvent B in 20 min, at a flow rate of 1 ml/min. In the case of the Nova-Pak columns, the gradient ran from 35 to 45% solvent B in 20 min, at a flow rate of 0.5 ml/min. Absorbance was monitored at 214 or 210 nm.

In the first HPLC runs 1-min fractions were collected and used for bioassays. In later runs only the active fractions were collected manually and used for the elucidation of the primary structure by automated sequencing and mass spectrometry and for structural confirmation by isolation on various HPLC supports.

3.7 Sequence determination and mass spectrometry

3.7.1 Removal of N-terminal pyroglutamate

Sequencing by the Edman method occurs by selective cleavage of the amino acid at the free amino terminal of the peptide, and leads to the determination of one amino acid after another. To use the Edman method for sequencing of AKH/RPCH family peptides, it is necessary to remove the blocked pyroglutamate residue that is present in all peptides of the AKH/RPCH family at the N-terminus. Purified, active peptide material was enzymically deblocked using L-pyroglutamate aminopeptidase (EC 4.4.19.3). The HPLC purified peptide (about 200-400 pmol) was taken up in 80 µl buffer (shaking vigorously for 1 min), and 20 µl pyroglutamate aminopeptidase (2.88 mg/ml buffer) added. The buffer (pH 8.0) consisted of:

Na_2HPO_4	100 mM
EDTA	10 mM
DTT	5 mM
Glycerol	5% v/v

The mixture was incubated at 37°C, and the reaction stopped by addition of an equal volume of a solution of 15% ACN containing 0.1% TFA.

It was necessary to optimise the incubation time (usually between 30 and 90 min) for each new enzyme batch in the following way: a quantity of any suitable AKH/RPCH peptide equivalent to around 0.2 AUFS was incubated with the enzyme and aliquots of 10 µl taken at various time intervals (e.g. 0, 15, 30, 45, 60 min etc.). The peak height or area of the deblocked and undigested peptides was measured by separating the peptides with RP-HPLC (see below) for each of these aliquots, so as to determine the incubation time that would maximise the yield of the deblocked product.

The deblocked peptide was separated by RP-HPLC from the undigested peptide, as well as any breakdown products, and buffer compounds. The same chromatographic system as described previously was used, with the separation achieved on a Nucleosil C18 column. A different gradient running from 33 to 53% solvent B in 40 min was applied. The deblocked peptide was manually collected and dried in a vacuum centrifuge.

3.7.2 Edman sequencing

The deblocked peptide was sent to the laboratory of Dr. Roland Kellner (EMBL, Heidelberg, Germany) where it was dissolved, spotted onto polybrene coated glass fibre discs and subjected to automated Edman degradation (model 477A; Applied Biosystems). The sequencer was connected to an on-line PTH-amino acid analyser (model 120A; Applied Biosystems). Sequencing reagents and solvents were from Applied Biosystems. Sequencing and PTH-analysis were carried out according to standard protocols.

3.7.3 Mass spectrometry

Mass spectra were acquired by Dr. Kellner and colleagues at EMBL using a Sciex API III mass spectrometer (Sciex, Thornhill, Canada), and later using a matrix-assisted laser desorption ionisation spectrometer (Bruker REFLEX, Bruker-Franzen, Bremen, Germany) (Hillenkamp *et al.*, 1991).

3.8 Chemicals, biochemicals and synthetic peptides

Radioactively labelled inulin ([^{3}H(G)]-inulin; 355.3 mCi/g) was obtained from DuPont. Ultima Gold XR scintillation fluid was used for radioactivity counting (Packard). Other chemicals were the highest purity available and obtained from Merck, BDH, Sigma and Saarchem. All biochemicals were obtained from Boehringer Mannheim, except the enzyme pyroglutamate aminopeptidase which was purchased from Sigma.

The hypertrehalosaemic peptide from *P. americana*, *Pea*-CAH-I, the adipokinetic peptide of *L. migratoria*, *Lom*-AKH-I, and the red pigment-concentrating hormone of *P. borealis* (*Pab*-RPCH) came from Peninsula Laboratories (Belmont, CA, USA). The adipokinetic peptides from *L. auripennis*, *Lia*-AKH, and *A. imperator*, *Ani*-AKH, as well as *P. inconspicuum* (*Psi*-AKH) were synthesised by Dr. Roland Kellner (EMBL, Heidelberg, Germany). The peptides were purified by RP-HPLC and their identities verified by amino acid analysis and mass spectrometry.

3.9 *In vitro* synthesis and incorporation of labelled ^{3}H-Phe

Radioactively labelled ^{3}H-Phe (5 µCi) was dissolved in 50 µl saline, and 7 CC from *O. julia* added. The composition of the saline solution (pH 7.2) was:

HEPES	10 mM
$CaCl_2$	5 mM
KCl	5 mM
$MgCl_2$	1 mM
NaCl	140 mM
$NaHCO_3$	4 mM
Sucrose	55 mM
Trehalose	50 mM
Penicillin/Streptomycin	0.18 mg/ml

The CC were incubated for 16 h at 25°C with constant gentle shaking. The glands were washed three times with saline solution free of ^{3}H-Phe and homogenised in 0.1% TFA with a sonifier (2 x 10 s.). After centrifugation at 12000 g for 5 min the

supernatant was dried down in a vacuum centrifuge. The dried material was dissolved in 25% solvent B and fractionated by RP-HPLC using the Nova-Pak phenyl column as described previously. The fluorescence of the eluate was monitored, and 20 s. fractions were manually collected. Scintillation fluid (4.5 ml) was added and the radioactivity in the fractions was measured by liquid scintillation counting using a Tri-Carb 460 (Packard) scintillation counter. The samples were counted twice for 5 min each.

3.10 Determination of haemolymph volume

The method according to Clegg and Evans (1961) was used. In brief, a trace amount of tritiated inulin contained in 1 µl water was injected into the dragon- or damselflies, as described previously. The amount of radioactivity of the solution to be injected was measured in triplicate for each set of experiments, and was ca. 36 487 counts/min. After 20 min, a 0.5 µl haemolymph sample was obtained and added to 4.5 ml scintillation fluid and assayed for radioactivity. The control was a blood sample taken from a dragonfly that had not been injected. The average CPM was used to determine the blood volume according to the formula:

$$\text{Blood vol.} = \frac{\text{[injected CPM]. [vol. of blood sample]}}{\text{[CPM of blood sample]}} - \text{[vol. injected]}$$

3.11 Flight experiments

Adult specimens of *O. julia* were left under funnels for at least 4 h at 26 ± 2°C prior to the experiments, to calm them down sufficiently. Haemolymph samples (0.5 µl) were then taken, and two dragonflies at a time forced to fly free in the room. As soon as they settled they were made to fly again, for exactly 30 min which was near the maximum time they could be induced to fly. Haemolymph samples (0.5 µl) were taken immediately before and after flight, and analysed for either total carbohydrates, lipids or the free amino acids Pro and Ala (as described below). The concurrent control consisted of dragonflies that were not provided with the opportunity to fly. Directly after haemolymph sampling, the dragonflies were sacrificed, and the flight muscle dissected as described below. The content of polysaccharides (mostly glycogen), Ala and Pro were determined in the flight muscles.

3.11.1 Preparation of extracts from flight muscles

Dragonflies were decapitated, the tip of the abdomen cut off, the abdomen opened ventrally and the gut removed. The thorax was opened ventrally and the flight muscles removed. The whole procedure took ca. 1.5 min. There was no discernible fat body tissue.

In locusts, the tip of the abdomen was cut off, the head twisted to detach it from the thorax, and with the gut still attached, removed by pulling from the anterior end. The thorax was opened ventrally, the fat body removed with paper towel and the flight muscles removed.

The flight muscles were frozen immediately in liquid nitrogen, and stored at -20°C. The flight muscles were ground to a fine powder with a mortar and pestle that had been pre-cooled with liquid nitrogen. The frozen powder was transferred to a pre-weighed centrifuge tube containing 1 M perchloric acid (PCA) which was adjusted to give a tissue/PCA ratio of 1:5, the mixture was sonicated twice for 10 s. and then centrifuged for 10 min at 8000 g at 4°C (RC5C, Sorval). The pellet was resuspended in PCA and the extraction procedure repeated. The combined supernatants were neutralised by adding 3 M K_2CO_3, the precipitated $KClO_4$ was spun down (10 min at 8000 g; 4°C) and the volume of the final extract measured. Extracts were stored at -20°C.

3.11.2 Determination of proline and alanine

Amino acids were quantified by means of HPLC using the Beckman System Gold as described previously, fitted with a Spherisorb ODS II-5µm column (diameter 4.6 mm, length 125 mm) with a 20 mm precolumn of the same material.

The amino acids were transformed to dansyl derivatives. A 25 µl sample was mixed with 100 µl $NaHCO_3$ solution (0.5 M, pH 8.5) and 100 µl dansyl chloride (6 mg/ml acetone). The mixture was incubated for 4 h at room temperature in the dark and then diluted by adding 800 µl $NaHCO_3$ solution.

The HPLC buffers were: (A) formic acid (50 mM), acetic acid (60 mM); (B) 35% 2-propanol, in solvent A. A linear gradient was used, increasing solvent B from 25 to 80% over 25 min at a flow rate of 1 ml/min. The injected volume was 100 or 200 µl, and the absorbance of the eluate was monitored at 254 nm. Standard solutions of Ala and Pro were dansylated as described above and used to identify the correct peaks as Ala and Pro by retention time, and to calibrate the measured peak areas.

3.11.3 Determination of glycogen

Glycogen determination was carried out by precipitation of the glycogen from an aliquot of PCA extract with 5 ml ethanol, to which 200 µl Na_2SO_4 (saturated) was added. The mixture was kept cold overnight and centrifuged for 20 min at 10 000 g at 4°C. The precipitate was redissolved in 1 ml water and 25 µl samples were analysed employing the anthrone method described previously.

3.12 Determination of enzyme activities

Freshly dissected muscles were pooled, weighed and disrupted in cold homogenisation medium with an Ultra-Turrax homogeniser (Janke-Kunkel). The homogenisation medium (pH 7.6) consisted of:

TRA-HCl	50 mM
EDTA	1 mM
DTE	0.1 mM

The homogenate was centrifuged at 14 000 g for 30 min at 4°C. The enzyme activities in the clarified supernatant were determined at 25°C in a 1 ml cuvette. All reactions were started by addition of the substrate. The enzyme assays were performed using two concentrations of extract to ensure proportionality.

Citrate synthase (CS) (EC 4.1.3.7) was determined according to Zebe and Gäde (1993). The rate of change of the absorbance at 412 nm was measured in an assay medium (pH 8.0) consisting of:

Tris-HCl	50 mM
DTNB	0.25 mM
Acetyl-CoA	0.05 mM
Oxaloacetate	0.05 mM

The following reactions measured the rate of oxidation of NADH by monitoring the change in absorbance at 340 nm.

3-Hydroxyacyl-CoA dehydrogenase (HOAD) (EC 1.1.1.35) was measured, modified after Bass *et al.* (1969). The assay medium (pH 7.0) consisted of:

TRA-HCl	100 mM
EDTA	5 mM
NADH	0.15 mM
Acetoacetyl-CoA	0.1 mM

Phosphofructokinase (PFK) (EC 2.7.1.11) was measured, modified after Meinardus-Hager and Gäde (1992), in an assay medium (pH 7.6) consisting of:

TRA-HCl	100 mM
ATP	1 mM
KCl	100 mM
$MgCl_2$	5 mM
NADH	0.15 mM
Fructose-6-phosphate	5 mM
Aldolase	1 U
Glycerine-3-phosphate dehydrogenase	3.9 U
Triosephosphate isomerase	11.3 U

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (EC 1.2.1.12) was determined, with modifications after Beenakkers (1969a), in an assay medium (pH 7.6) consisting of:

TRA-HCl	50 mM
ATP	1.5 mM
EDTA	5 mM
Mercaptoethanol	2.4 mM
$MgSO_4$	3.3 mM
NADH	0.15 mM
3-Phosphoglycerate	7 mM
3-Phosphoglycerokinase	200 U

Lactate dehydrogenase (LDH) (EC 1.1.1.27) was determined according to Beenakkers (1969a), in an assay medium (pH 7.6) consisting of:

TRA-HCl	50 mM
EDTA	5 mM
NADH	0.15 mM
Pyruvate	2.4 mM

Pyruvate kinase (PK) (EC 2.7.1.40) was determined, with modifications, according to Simon and Robin (1972), in an assay medium (pH 7.6) consisting of:

TRA-HCl	100 mM
ADP	2 mM
KCl	20 mM
$MgSO_4$	4 mM
NADH	0.15 mM
Lactate dehydrogenase	8 U
Phosphoenolpyruvate	10 mM

The total activity of glycogen phosphorylase (EC 2.4.1.1) was measured in the direction of glycogen breakdown, by monitoring the rate of reduction of NADP at 340 nm. The assay medium (pH 7.0) was essentially the same as described in Ziegler *et al.* (1979), and consisted of:

TRA-HCl	50 mM
AMP	1.5 mM
DTT	2.6 mM
EDTA	2 mM
Glucose-1,6-bisphosphate	1.5 µM
Glycogen	10 mg/ml
Imidazole	5 mM
KH_2PO_4	60 mM
Magnesium acetate	4.8 mM
NADP	0.4 mM
Phosphoglucomutase	0.24 U
Glucose-6-phosphate dehydrogenase	0.85 U

3.13 Isolation of mitochondria from flight muscles

3.13.1 Homogenisation and substrate oxidation

Flight muscles (ca. 500 mg) were transferred into 10 ml ice cold isolation medium (pH 7.2) consisting of:

HEPES	10 mM
BSA	1%
EDTA	2 mM
EGTA	1 mM
Sucrose	250 mM

The flight muscles were homogenised with three to four passes of a tight-fitting hand-held Potter-Elvehjem homogeniser on ice. The homogenate was centrifuged at 100 g for 10 min at 4°C. The supernatant was centrifuged at 9000 g for 10 min at 4°C.

The pellet was resuspended in fresh isolation medium, centrifuged and the pellet resuspended in a small volume of isolation medium.

The mitochondrial suspension was added to 1 ml of an assay medium (pH 7.2) consisting of:

HEPES	10 mM
ADP	0.5 mM
BSA	0.15%
Cytochrome C	0.2 mg/ml
EDTA	1 mM
KCl	150 mM
KH_2PO_4	10 mM
$MgCl_2$	5 mM

The mitochondrial suspension was added in a volume of 25 or 50 µl, to test for linearity in the assay. The oxygen consumption rate was measured polarographically (model 5300, YSI) at 25°C, and expressed as change in the percentage of O_2 saturation, using air-saturated distilled water as the reference. The reaction was started by addition of substrate:

α-Glycerol-3-phosphate (with 1 mM $CaCl_2$)	5 mM
Malate (with 0.1 mM pyruvate)	6 mM
Palmitoyl-carnitine	20 µM
Pro	20 mM
Pyruvate (with 0.1 mM malate)	5.7 mM
Succinate	5 mM

3.13.2 Cytochrome oxidase activity

The determination of Cytox activity was according to Rafael (1985) in 1 ml of an assay medium (pH 7.2) consisting of:

KH_2PO_4	50 mM
Ascorbate	7 mM
Cytochrome C	85 µM
EDTA	1 mM
TMPD	0.7 mM

The rate of O_2 consumption of a mitochondrial solution was measured polarographically after adding in a volume of 5 and 10 µl, to test for proportionality. The basal oxygen consumption rates due to autoxidation of TMPD in the presence of ascorbate was subtracted.

4. RESULTS

4.1 Adipokinetic/hypertrehalosaemic factors in the CC

In the first series of experiments crude methanolic extracts of CC from some common dragon- and damselflies were prepared. Aliquots of these CC extracts were injected into locusts, and sometimes cockroaches, to test for the presence of hyperlipaemic and/or hypertrehalosaemic factors, in a rapid, and physiologically relevant way (Gäde, 1990c).

Crude methanolic extracts prepared from CC of *O. julia* (0.2 gland equivalents) produced a hyperlipaemic response when injected into acceptor locusts (Table III). Extracts of CC of *A. imperator* (0.2 gland equivalents) as well as of *P. inconspicuum* (1.6 gland equivalents) elicited increases in haemolymph lipids in locusts and carbohydrates in cockroaches, compared with control injections of water. The maximal possible responses in locusts and cockroaches is obtained by conspecific injection of 0.1 gland equivalent of the CC of each bioassay species. The increases elicited by injection of 0.2 gland equivalents of *O. julia* amounted to ca. 38% of the maximum possible lipid release (Table III). In the case of *A. imperator* 0.2 gland equivalents elicited 52% of the maximal lipid release and 72% of the carbohydrate release. The damselfly material (1.6 gland equivalents) amounted to ca. 40% for lipid release and 62% for carbohydrate release.

Adipokinetic and/or hypertrehalosaemic factors are also present in CC of the larvae. The maximal possible adipokinetic response (100%) was achieved in locusts when injected with a CC extract from *A. imperator* larvae (0.7 gland equivalents), and a CC extract from larvae of a combination of *C. erythraea* and *O. julia* (2.9 gland equivalents) (Table III).

Having shown that the CC of the odonates under investigation contain adipokinetic and/or hypertrehalosaemic substances, the activity of such extracts on haemolymph metabolites in the dragonflies themselves were investigated.

Table III. Adipokinetic and hypertrehalosaemic activity of extracts from CC

Acceptor insect:	*L. migratoria*				*P. americana*			
Treatment	Blood lipids (mg/ml)				Blood carbohydrates (mg/ml)			
	n	Before	After	Difference	n	Before	After	Difference
(A) Adult dragonflies								
Control; water	8	10.8 ± 2.2	10.8 ± 3.1	0.0 ± 2.4				
0.2 CC equivalents of *O. julia*	8	8.0 ± 1.8	21.4 ± 8.3	13.4 ± 7.0***				
0.1 CC equivalents of *L. migratoria*	8	9.8 ± 3.0	45.5 ± 9.7	35.7 ± 9.0***				
Control; water	8	10.1 ± 1.7	11.7 ± 2.9	1.6 ± 2.1	10	17.1 ± 4.5	19.6 ± 5.0	2.5 ± 2.9
0.2 CC equivalents of *A. imperator*	8	11.4 ± 3.9	35.1 ± 6.1	23.7 ± 3.8***	8	19.7 ± 2.9	45.8 ± 7.4	26.1 ± 6.9***
0.1 CC equivalents of *L. migratoria*	8	10.4 ± 1.9	55.9 ± 4.8	45.5 ± 4.1***				
0.1 CC equivalents of *P. americana*					10	17.1 ± 3.1	53.4 ± 10.6	36.3 ± 10.6***
(B) Dragonfly larvae								
Control; water	6	10.1 ± 1.7	11.7 ± 2.9	1.6 ± 2.1				
2.9 CC equivalents of *C. erythraea* & *O. julia*	6	17.7 ± 5.9	57.6 ± 15.9	39.9 ± 14.7**				
0.7 CC equivalents of *A. imperator*	6	21.9 ± 3.4	61.0 ± 17.5	39.1 ± 16.0**				
0.1 CC equivalents of *L. migratoria*	6	20.0 ± 2.2	56.1 ± 23.4	36.1 ± 23.5†				
(C) Adult damselfly								
Control; water	8	10.1 ± 1.7	11.8 ± 2.9	1.7 ± 2.1	7	17.8 ± 3.8	19.6 ± 3.9	1.8 ± 2.0
1.6 CC equivalents of *P. inconspicuum*	6	9.5 ± 3.0	26.8 ± 11.6	17.3 ± 9.2*	9	17.1 ± 2.5	35.8 ± 11.5	18.7 ± 9.7***
0.1 CC equivalents of *L. migratoria*	8	10.4 ± 1.9	55.9 ± 4.8	45.5 ± 4.1***				
0.1 CC equivalents of *P. americana*					7	15.8 ± 3.0	48.1 ± 12.7	32.3 ± 11.3***

Locusts and cockroaches were injected with crude methanolic extracts of CC from the dragonflies, *O. julia* and *A. imperator*: adults (A), larvae (B), and adult damselflies, *P. inconspicuum* (C). Values are mean ± SD of the concentration in the haemolymph of total lipids in adult male locusts and total carbohydrates in adult male cockroaches. The significance of the difference between values before and after injection (120 min for cockroaches; 90 min for locusts) is indicated by † $p<0.05$, * $p<0.01$, ** $p<0.005$ and *** $p<0.001$.

For the initial pilot experiments *A. imperator* was chosen because of its size (cf. Table XV p. 57). Difficulty was experienced confining the adult dragonflies in such a way as not to elicit constant escape behaviour, injecting them without inflicting unnecessary damage, and taking haemolymph samples. It is probable that in some cases the gut of the insects had been punctured during injection. In an initial experiment the

lipid concentration in the haemolymph of the water-injected control group was increased by 8.0 ± 3.9 mg/ml (n=8). However, injection of conspecific CC extract led to an increase in the concentration of haemolymph lipids (14.7 ± 7.4 mg/ml; n=11), that was significantly higher ($p<0.05$) than the increase in blood lipids in the control group. No effect could be demonstrated on the concentration of total carbohydrates in the haemolymph: the change in carbohydrates in the haemolymph of the water-injected group (-0.3 ± 2.6 mg/ml; n=8) was not significantly different from the CC-injected group (0.7 ± 2.3 mg/ml; n=5).

In subsequent experiments the conspecific adipokinetic effect was demonstrated more clearly. This was attributed to increased skill in handling the dragonflies, minimising escape behaviour and wing flapping, and inflicting less damage whilst taking haemolymph samples. An increase of the initial concentration of haemolymph lipids by ca. 57% and 96%, was measured for adults of *A. imperator* and *O. julia*, respectively, after injection of conspecific CC extract (Table IV). Conspecific injection of one gland equivalent into *P. inconspicuum* resulted in a significant elevation of both the haemolymph lipids (77%) and carbohydrates (34%), compared with the control group. No significant hypertrehalosaemic or adipokinetic effect could be demonstrated after injection of conspecific CC extract into the larvae of *O. julia* and *C. erythraea* (Table IV).

All the bioassays with adult *O. julia* and *A. imperator* were performed at 10°C. This temperature had been chosen to allow for easier handling of the dragonflies (Gäde, 1990a). It was therefore of interest to determine the influence of this temperature on the magnitude of the adipokinetic effect. A simple test was done on the standard bioassay animal, *L. migratoria.* Locusts from the same batch were injected with extract of conspecific CC. One experiment was performed at a temperature of 10°C, and the other at 25°C. There was virtually no change in the concentration of haemolymph lipids in the control group that were kept at 10°C for the duration of the experiment (0.8 ± 1.2 mg/ml, n=8), nor in the control group at 25°C (1.5 ± 1.9 mg/ml, n=8). The increase in the concentration of haemolymph lipids was significantly lower ($p<0.001$) in the CC-injected group of locusts at 10°C (9.0 ± 4.5 mg/ml; n=7) than in the group at 25°C (30.7 ± 8.1 mg/ml; n=8).

Table IV. Conspecific adipokinetic and hypertrehalosaemic effect of Odonata CC

Acceptor insect:	Blood lipids (mg/ml)				Blood carbohydrates (mg/ml)			
Treatment	n	Before	After	Difference	n	Before	After	Difference
(A) Adult dragonflies								
O. julia								
Control; water	5	31.9 ± 14.1	34.8 ± 15.8	2.9 ± 2.1	10	4.1 ± 2.3	4.4 ± 3.4	0.3 ± 1.9
1.0 CC equivalents	6	20.4 ± 4.6	40.0 ± 4.7	19.6 ± 3.2***	6	3.9 ± 1.3	3.6 ± 1.8	-0.3 ± 1.1
A. imperator								
Control; water	7	55.3 ± 23.6	56.2 ± 22.1	0.9 ± 5.8	7	2.0 ± 1.0	2.6 ± 1.1	0.6 ± 0.5
1.0 CC equivalents	4	51.9 ± 18.6	81.7 ± 23.9	29.8 ± 12.0†	4	2.1 ± 1.5	4.0 ± 2.6	1.9 ± 1.3
(B) Dragonfly larvae								
C. erythraea & *O. julia*								
Control; water	26	7.3 ± 4.1	6.6 ± 3.7	-0.7 ± 1.3	24	3.8 ± 1.3	4.5 ± 2.1	0.7 ± 1.8
1.0 CC equivalents	24	10.8 ± 3.9	10.5 ± 4.0	-0.3 ± 1.3	24	4.7 ± 2.0	5.6 ± 1.4	0.9 ± 1.9
A. imperator								
Control; water	10	11.7 ± 1.7	12.1 ± 2.6	0.4 ± 2.7	10	4.1 ± 1.5	5.2 ± 2.4	1.1 ± 3.3
1.0 CC equivalents	14	8.0 ± 4.7	8.2 ± 4.2	0.2 ± 2.1	14	3.7 ± 1.4	5.1 ± 2.4	1.4 ± 2.6
(C) Adult damselfly								
P. inconspicuum								
Without injection	17		16.1 ± 5.8		22		6.4 ± 2.5	
Control; water	33		14.6 ± 4.3		36		5.9 ± 2.6	
1.0 CC equivalents	19		25.8 ± 6.2***		22		7.9 ± 2.5***	

Adults (A) and larvae (B) of the dragonflies *O. julia* and *A. imperator*, and adult damselflies *P. inconspicuum* (C) were injected with a crude methanolic extract of conspecific CC. Values are mean ± SD of the concentration in the haemolymph of total lipids and carbohydrates. The significance of the difference between values before and 90 min after injection is indicated by † $p<0.05$, * $p<0.01$, ** $p<0.005$, and *** $p<0.001$. In the case of the damselfly the significance of differences between the control and experimental group is given.

4.2 Initial peptide purification and sequencing

The aim of this phase of the project was to establish whether there were any AKH peptides other than *Lia*-AKH in the CC of Odonata. Methanolic extracts of CC were prepared from the Odonata under investigation. The extracts were fractionated using RP-HPLC and the retention times compared with that of synthetic *Lia*-AKH. The biologically active fractions were determined using the locust bioassay, and the activity confirmed using a conspecific bioassay where possible.

Table V. Deblocking of AKH peptides with pyroglutamate aminopeptidase

	Lia-AKH (-pGlu)		*Lia*-AKH		
Time (min)	Rt (min)	Area	Rt (min)	Area	Yield (%)
0	-	-	19.52	6.43	
30	16.43	4.00	19.50	2.29	62.2
60	16.46	4.56	19.54	1.13	70.9
120	16.55	4.63	19.61	0.68	72.0

The N-terminal pyroglutamate residue of synthetic *Lia*-AKH was enzymically removed, resulting in the deblocked *Lia*-AKH (-pGlu), after incubation with pyroglutamate aminopeptidase at 37°C for various lengths of time. Aliquots were subjected to RP-HPLC using chromatographic conditions as described in Fig. 2; the gradient ran from 33 to 53% B in 40 min. Peak areas were integrated using the Beckman System Gold and are in arbitrary units.

All known members of the AKH/RPCH family have an N-terminal pyroglutamyl residue (Gäde, 1990c). Assuming the presence of such a residue, aliquots of isolated active material from dragonflies were deblocked using pyroglutamate aminopeptidase (Section 3.7.1; p. 18). An example is given of optimising the incubation time with the enzyme, using synthetic *Lia*-AKH (Table V). The deblocked material was purified using RP-HPLC and was eluted earlier than the native material. The deblocked peptides were subjected to automated Edman sequencing.

Orthetrum julia falsum (Anisoptera: Libellulidae)

A methanolic extract of CC from *O. julia* adults were applied to a RP-HPLC C18 column. The 1 min fractions that were collected were tested for the presence of adipokinetic compounds using the locust bioassay. The change in haemolymph lipids after injection of the particular fraction into locusts is depicted below the chromatograms in the form of histograms (Fig. 2A) and bar charts (Fig. 2C).

The active peak had the same retention time in HPLC runs as synthetic *Lia*-AKH. More than one UV absorbance peak was collected into a fraction that was shown to have biological activity (Fig. 2 A & B). In each of these cases only one UV peak had a clear fluorescence peak associated with it. The peaks were collected separately in later HPLC runs (Fig. 2 C) and tested in the locust bioassay. The retention time of the active compound was identical (12.2 min) to that of synthetic *Lia*-AKH (Fig. 2 D).

Active peak material (1 gland equivalent) was injected into adult *O. julia* and resulted in a doubling of the concentration of lipids in the haemolymph (Table X).

The bioactive compound detected in CC of *O. julia* and *C. erythraea* larvae (Table III) was isolated using HPLC. An equivalent of 0.5 glands injected per locust led to an increase of 4.8 ± 1.9 mg/ml (n=6) ($p<0.002$) in the concentration of the haemolymph lipids, compared with a control group which had an insignificant increase of 1.7 ± 2.3 mg/ml (n=5). This bioactive compound had an indistinguishable retention time (13.32 min) to synthetic *Lia*-AKH (13.28 min).

Active material was subjected to enzymatic deblocking using pyroglutamate aminopeptidase and subjected to automated Edman sequencing. The residues determined were Val-Asn-Phe-Thr-Pro-Ser-Trp. The first amino acid of the peptide must be pGlu as it was enzymically removed to enable the Edman method to degrade the peptide from its now free N-terminus. As all AKH/RPCH peptides have an amidated C-terminus this was also assumed to be true for this peptide (cf. section 4.3; p. 42) (Gäde, 1990c). This sequence is identical to that of *Lia*-AKH.

Crocothemis erythraea, *Trithemis dorsalis*, *Pantala flavescens* (Anisoptera: Libellulidae)

Corpora cardiaca of the above libellulids were screened in the same way, with 1 min fractions collected during RP-HPLC fractionation. One active fraction (Table VI), corresponding to both a UV and fluorescence peak, was co-eluted with *Lia*-AKH. This identified the active compound to be *Lia*-AKH.

Ceratogomphus pictus (Anisoptera: Gomphidae)

All fractions collected after HPLC purification of CC of *C. pictus* were also tested to identify fractions containing adipokinetic activity (Table VI). The active fraction (no. 13), corresponding to both a UV and fluorescence peak, was co-eluted with *Lia*-AKH. This identified the active compound to be *Lia*-AKH.

Anax speratus (Anisoptera: Aeshnidae)

A methanolic extract of 2 CC of *A. speratus* was applied to a C18 RP-HPLC column. The elution profile shows only a few UV absorbance, as well as fluorescence peaks (Fig. 3). When certain fractions (numbered 1 to 4) were measured for hyperlipaemic activity in locusts, only peak 4 was active (Table VII).

The material of peak 4 was clearly associated with both a UV and fluorescence peak. Comparison of the retention time of the active fractions of *A. speratus* with *Lia*-AKH revealed that they were not co-eluted. *Lia*-AKH (12.9 min) was eluted later than the active material from *A. speratus* (12.3 min). The active material from *A. speratus* was also not co-eluted with the synthetic AKH/RPCH members, *Pab*-RPCH (17.5 min), *Lom*-AKH-I (15.2 min), and *Pea*-CAH-I (11.1 min) (Fig. 3).

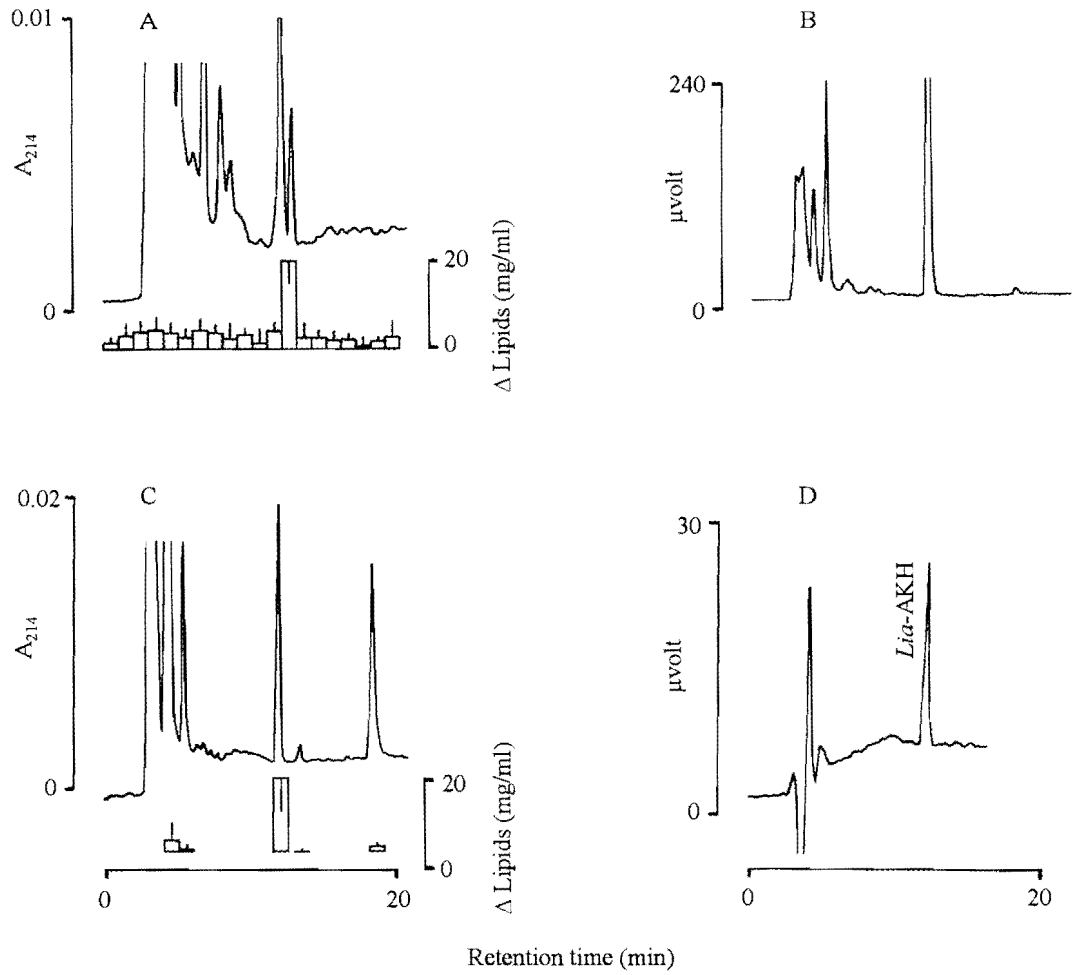


Fig. 2. RP-HPLC isolation of adipokinetic compounds from CC of *O. julia*

Crude methanolic extracts of CC of *O. julia* (A, B & C), and synthetic *Lia*-AKH (D) were applied to a Nucleosil C18 100 column which was eluted with a linear gradient of 0.11% TFA (solvent A) and 0.1% TFA in 60% ACN (solvent B). The gradient ran from 43 to 53% solvent B during 20 min. at a flow rate of 1 ml/min. UV absorbance was monitored at 214 nm (A & C). Fluorescence was measured at 276 nm (excitation) and 350 nm (emission) (B & D) and expressed as μvolt detector output as explained in section 3.6; p. 17. The histogram represents the mean and SD of differences of haemolymph lipid concentration in at least 5 locusts injected with 1 min. fractions, at a concentration of 1 CC equivalent, not taking any losses into account.

Table VI. Adipokinetic effect of RP-HPLC fractions

Fraction	Before	After	Difference	n	Before	After	Difference	n
	Trithemis dorsalis				*Crocothemis erythraea*			
1	7.3 ± 0.8	9.3 ± 1.6	1.9 ± 1.0	6	9.7 ± 2.0	11.0 ± 3.3	1.4 ± 2.5	6
2	8.6 ± 2.2	10.7 ± 3.2	2.1 ± 1.5	6	9.5 ± 2.2	8.9 ± 2.2	-0.6 ± 0.7	6
3	8.1 ± 1.3	9.3 ± 1.2	1.2 ± 0.7	6	9.1 ± 2.3	10.4 ± 3.1	1.4 ± 1.5	6
4	8.6 ± 2.0	10.2 ± 4.1	1.6 ± 2.3	6	9.7 ± 2.1	10.5 ± 3.2	0.9 ± 1.4	6
5	8.2 ± 1.4	9.5 ± 1.7	1.3 ± 0.9	5	11.8 ± 4.0	11.8 ± 4.4	0.0 ± 1.7	6
6	8.2 ± 0.9	9.6 ± 1.9	1.4 ± 1.2	6	9.5 ± 1.3	10.6 ± 1.9	1.1 ± 1.4	6
7	7.6 ± 1.8	10.0 ± 3.3	2.4 ± 2.2	6	11.8 ± 4.3	12.4 ± 5.6	0.5 ± 2.0	6
8	7.1 ± 0.7	8.7 ± 2.3	1.6 ± 1.8	6	14.6 ± 4.8	16.9 ± 6.9	2.3 ± 3.1	6
9	7.5 ± 2.1	8.7 ± 3.5	1.2 ± 1.7	6	10.0 ± 1.6	11.1 ± 0.6	1.2 ± 1.4	5
10	6.9 ± 1.1	8.0 ± 1.9	1.0 ± 1.6	6	12.7 ± 3.7	15.1 ± 6.4	2.5 ± 3.2	6
11	8.5 ± 1.3	9.3 ± 2.1	0.7 ± 1.6	6	13.3 ± 1.5	14.7 ± 1.1	1.4 ± 1.1	6
12	7.2 ± 1.7	9.6 ± 4.1	2.4 ± 4.0	6	13.3 ± 5.3	15.4 ± 5.6	2.1 ± 1.5	6
13	**8.5 ± 2.6**	**29.0 ± 3.1**	**20.5 ± 1.9**	**6**	**15.4 ± 6.2**	**38.0 ± 10.4**	**22.6 ± 6.1**	**6**
14	8.9 ± 2.0	12.9 ± 5.1	4.0 ± 4.1	6	10.6 ± 2.6	12.8 ± 4.7	2.2 ± 3.2	6
15	11.9 ± 3.2	15.3 ± 5.2	3.3 ± 2.9	7	8.6 ± 2.3	10.9 ± 1.8	2.2 ± 1.4	6
16	12.1 ± 2.0	14.7 ± 4.5	2.6 ± 3.7	7	9.6 ± 3.0	13.7 ± 6.5	4.1 ± 5.1	6
17	10.8 ± 2.9	13.2 ± 6.7	2.4 ± 4.3	6	8.7 ± 1.4	9.8 ± 1.7	1.0 ± 0.8	6
18	10.5 ± 1.2	15.5 ± 5.7	5.0 ± 4.6	8	9.6 ± 2.2	12.7 ± 2.2	3.1 ± 1.0	6
19	10.7 ± 1.3	13.3 ± 3.3	2.6 ± 2.5	6	9.0 ± 1.9	10.2 ± 2.6	1.2 ± 1.1	6
20	12.2 ± 2.9	14.8 ± 4.3	2.5 ± 2.3	8				
	Ceratogomphus pictus				*Pantala flavescens*			
1	15.7 ± 8.1	18.6 ± 7.0	2.9 ± 3.0	6	12.7 ± 2.8	15.7 ± 5.9	3.0 ± 3.8	6
2	11.9 ± 3.9	14.4 ± 4.1	2.5 ± 3.7	6	9.0 ± 2.3	9.8 ± 2.3	0.8 ± 1.7	6
3	13.6 ± 7.1	14.5 ± 9.3	0.9 ± 3.1	5	10.9 ± 1.1	13.4 ± 2.9	2.5 ± 2.2	6
4	9.6 ± 1.6	10.6 ± 2.4	1.0 ± 1.2	6	10.1 ± 2.5	13.5 ± 3.4	3.3 ± 1.6	5
5	15.4 ± 6.1	17.6 ± 6.6	2.3 ± 1.9	6	9.5 ± 2.6	13.2 ± 6.4	3.8 ± 4.2	6
6	10.2 ± 0.7	12.4 ± 2.7	2.2 ± 2.6	6	10.5 ± 1.5	12.9 ± 1.9	2.4 ± 1.8	6
7	9.3 ± 1.3	9.9 ± 1.0	0.6 ± 0.9	6	10.5 ± 1.5	12.9 ± 1.9	2.4 ± 1.8	6
8	12.3 ± 5.5	13.1 ± 6.3	0.8 ± 1.4	6	7.8 ± 1.5	8.6 ± 2.3	0.8 ± 1.0	5
9	9.7 ± 1.2	11.5 ± 2.9	1.8 ± 2.2	6	7.9 ± 1.7	9.4 ± 1.4	1.4 ± 1.2	6
10	8.0 ± 2.4	9.4 ± 3.2	1.4 ± 1.4	6	8.0 ± 1.4	10.3 ± 3.3	2.2 ± 2.2	6
11	11.0 ± 4.8	11.6 ± 5.3	0.6 ± 1.7	5	8.2 ± 0.9	8.5 ± 1.0	0.2 ± 0.6	6
12	7.6 ± 1.6	8.3 ± 1.9	0.6 ± 0.8	6	9.1 ± 1.1	10.4 ± 2.3	1.2 ± 1.2	6
13	**8.4 ± 1.8**	**25.3 ± 7.3**	**16.9 ± 5.7**	**5**	**10.1 ± 3.2**	**40.6 ± 9.8**	**30.4 ± 6.8**	**6**
14	8.2 ± 1.7	9.3 ± 2.2	1.1 ± 1.1	6	7.5 ± 1.9	9.1 ± 2.8	1.6 ± 1.0	6
15	9.0 ± 0.9	10.9 ± 3.0	1.9 ± 2.6	6	9.1 ± 1.9	11.4 ± 1.9	2.3 ± 1.2	5
16	8.5 ± 3.0	10.4 ± 5.4	1.9 ± 3.6	6	13.8 ± 2.7	19.8 ± 6.8	6.0 ± 4.6	6
17	5.8 ± 0.8	6.2 ± 1.1	0.4 ± 0.8	6	13.5 ± 2.1	17.4 ± 2.4	3.9 ± 1.0	6
18	7.4 ± 1.5	7.6 ± 1.5	0.2 ± 0.6	6	12.0 ± 1.4	16.2 ± 1.6	4.2 ± 0.9	6
19	7.8 ± 3.7	7.5 ± 3.0	-0.3 ± 0.9	6	10.6 ± 2.3	13.6 ± 3.1	3.0 ± 1.2	6
20	6.7 ± 1.7	6.6 ± 1.3	0.0 ± 0.7	6				

Locusts were injected with 1 CC equivalent of 1 min fractions collected during RP-HPLC, using the same chromatographic conditions as described in Fig. 2. Values are mean ± SD of the concentration in the haemolymph of lipids. Bold text indicates fractions with adipokinetic activity.

Table VII. Adipokinetic activity of RP-HPLC peaks of *A. speratus* CC

Fraction	Before	After	Difference	n
Control	11 ± 1.0	13.7 ± 2.8	2.4 ± 2.0	6
Peak 1	8.6 ± 2.5	10.4 ± 2.4	1.8 ± 1.7	6
Peak 2	10 ± 1.8	12.1 ± 3.6	1.8 ± 3.7	6
Peak 3	9.4 ± 2.1	11.5 ± 3.6	2.1 ± 3.4	6
Part of Peak 4	9.8 ± 1.8	15.7 ± 4.5	5.9 ± 3.4†	5

Locusts were injected with peaks collected during RP-HPLC of CC from *A. speratus* (Fig. 3). Values are mean ± SD of the concentration of total lipids in the haemolymph of locusts. The significance of the difference before and 90 min after injection is given by † $p<0.05$.

Anax imperator

During purification of a crude methanolic CC extract of *A. imperator* using RP-HPLC, a compound with both a UV and fluorescence peak, was eluted at an identical retention time to the adipokinetic compound detected in *A. speratus*. This compound had adipokinetic activity when injected into acceptor locusts, significantly increasing the lipid concentration in the haemolymph by 12.6 ± 6.5 mg/ml (n=6) ($p<0.01$), compared with a control group where the haemolymph lipids changed by 2.3 ± 3.3 mg/ml (n=6). An aliquot of this fraction representing 0.5 CC equivalent was also injected into *A. imperator* and significantly elevated the concentration of lipids in the haemolymph by 11.8 ± 2.3 mg/ml, compared with a change of 0.9 ± 5.8 mg/ml in the control group ($p<0.001$). Injection of this active fraction had no effect on the concentration of carbohydrates in the haemolymph, changing the concentration by -0.6 ± 0.7 mg/ml compared with 0.6 ± 0.5 mg/ml in the control group (Table X, p. 45).

It was concluded that *A. imperator* and *A. speratus* contain a novel AKH/RPCH family peptide, and hence gland material was processed via this single-step purification method to obtain sufficient material for structural elucidation. The residues determined were Val-Asn-Phe-Ser-Pro-Ser-Trp, with an N-terminal pGlu, and C-terminal amide assumed. The sequence is that of a novel peptide of the AKH/RPCH family, differing from *Lia*-AKH in one amino acid residue, at position 5 where there is a Thr to Ser substitution. As this peptide had been sequenced from fractions containing adipokinetic activity in the native *A. imperator*, the peptide was named *Ani*-AKH according to the nomenclature proposed by Raina and Gäde (1988).

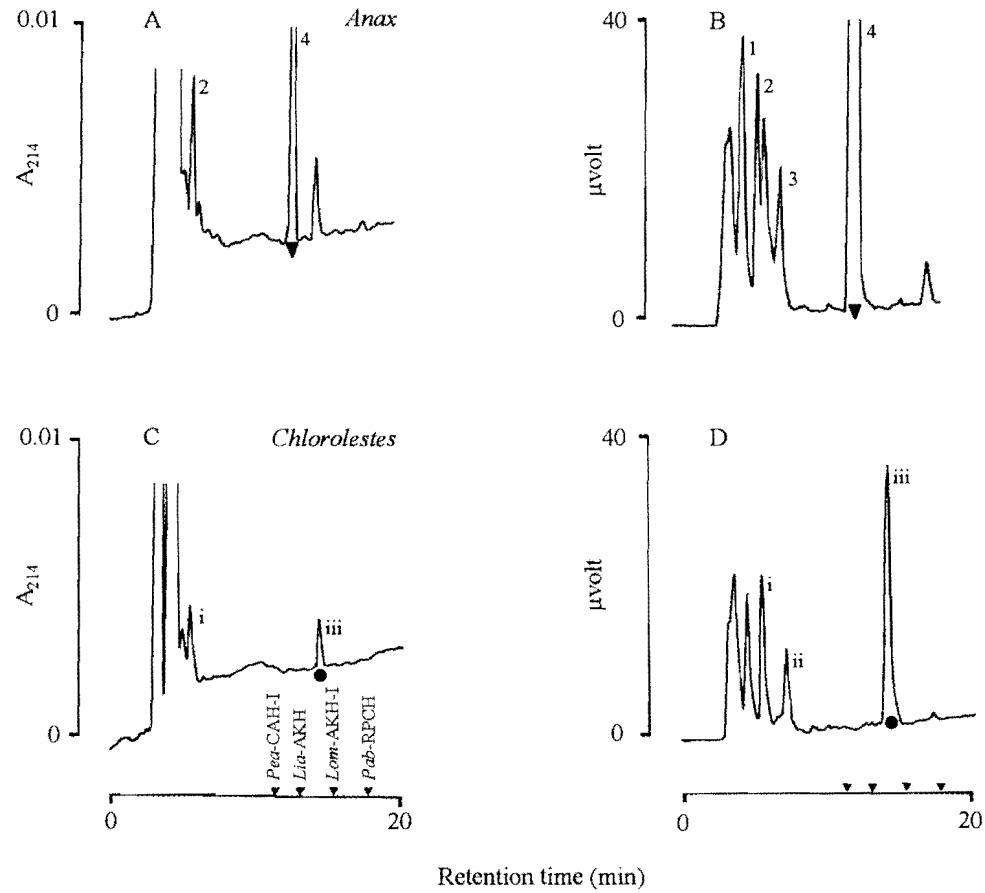


Fig. 3. RP-HPLC separation of CC from *A. speratus* and *C. tessellata*

Methanolic extracts of CC from *A. speratus* (A & B) and *C. tessellata* (C & D) were subjected to RP-HPLC, using a column and solvent system as described in Fig. 2. UV absorbance at 214 nm (A & C) and fluorescence (excitation 276 nm, emission 350 nm) (B & D) was monitored. The retention times of *Pea*-CAH-I, *Lia*-AKH, *Lom*-AKH-I, and *Pab*-RPCH are indicated. Peak 4 from *A. speratus* and peak iii from *C. tessellata* had adipokinetic activity when tested in locusts (Table VII and Table VIII, respectively).

Table VIII. Adipokinetic activity of RP-HPLC peaks of *C. tessellata* CC

	Before	After	Difference	n
Peak i	8.9 ± 1.4	10.2 ± 2.3	1.3 ± 1.4	6
Peak ii	11.6 ± 1.3	13.5 ± 3.1	1.9 ± 3.0	6
Peak iii	12.2 ± 4.2	17.4 ± 5.4	5.2 ± 2.2*	5

Locusts were injected with fractions collected during RP-HPLC of CC from *C. tessellata* (Fig. 3 C & D). Values are mean ± SD of the concentration of lipids in the haemolymph. The significance of the difference before and 90 min after injection is given by * $p<0.01$.

Chlorolestes tessellata (Zygoptera: Chlorolestidae)

An extract of 3 CC of the damselfly *C. tessellata* was fractionated using RP-HPLC (Fig. 3 C & D). A compound with both a UV and fluorescence peak, was eluted later (at 14.4 min) than both *Lia*-AKH (12.9 min) and the active fraction from *A. speratus* (12.3 min) (labelled 4 in Fig. 3 A & B) (*Ani*-AKH). When this fraction was tested in the locust bioassay it was found to have adipokinetic activity. The active compound was also not co-eluted with the synthetic AKH peptides *Pab*-RPCH (17.5 min), *Lom*-AKH-I (15.2 min), or *Pea*-CAH-I (11.1 min). The preliminary results indicated that there was a third AKH peptide present in the Odonata, other than *Lia*-AKH and *Ani*-AKH. As *C. tessellata* are very rare, it was impossible to obtain sufficient material to sequence this peptide, and another source had to be found.

Pseudagrion inconspicuum and *Ischnura senegalensis* (Zygoptera: Coenagrionidae)

A methanolic extract from a mixture of CC from *P. inconspicuum* (ca. 85% of the sample) and *I. senegalensis* was fractionated by RP-HPLC (Fig. 4). There were only a few distinct absorbance peaks at 214 nm. When each 1 min fraction was tested for adipokinetic activity in locusts, only fractions 13 and 14 were active (Fig. 4 A). This activity corresponds with the UV absorbance peak at 12.7 min, that also showed a clear fluorescence signal. No corresponding fluorescence was observed for the UV peak at 16.3 min; it was also not active in the locust bioassay. An aliquot of the peak fraction material at 12.7 min, representing one gland equivalent, was also injected into the damselfly *P. inconspicuum*. The concentration of the haemolymph lipids was

doubled when compared with control injections (Table X; p. 45). The effect of this fraction on the concentration of carbohydrates was not determined.

The elution pattern of the active compound was similar to the one observed earlier after fractionation of the CC from *C. tesselata*. (Fig. 3 C & D). When *P. inconspicuum* and *I. senegalensis* CC were fractionated, it was observed that the active fraction was eluted later (at 12.7 min) than synthetic *Ani*-AKH (10.7 min) and *Lia*-AKH (11.9 min) (Fig. 4). It was therefore concluded that *P. inconspicuum* and *I. senegalensis* contain a novel AKH family peptide. Sufficient gland material was purified, deblocked using pyroglutamate aminopeptidase, and subjected to Edman sequencing. The amino acid residues determined were Val-Asn-Phe-Thr-Pro-Gly-Trp, and including the N-terminal pGlu residue and assumed C-terminal amide, was identified as a novel AKH/RPCH peptide, differing from *Lia*-AKH only in the identity of the amino acid at position 7, where a Ser to Gly substitution is observed. As this peptide had been sequenced from fractions with adipokinetic activity in the native *P. inconspicuum*, the novel peptide was named *Psi*-AKH (Raina and Gäde, 1988). This sequence had been established from a mixture of *P. inconspicuum* and *I. senegalensis*, therefore for confirmation an extract prepared from CC of *I. senegalensis* was applied to a Nucleosil 100 C18 column for RP-HPLC. A fraction, associated with a UV peak (fluorescence was not monitored), was eluted at an identical retention time (11.8 min) to the active fraction from *P. inconspicuum* (11.8 min), as well as to synthetic *Psi*-AKH (11.7 min). This fraction significantly increased the lipid concentration in the haemolymph of locusts by 13.2 ± 4.4 mg/ml (n=6) ($p<0.001$) upon injection, compared with a control group in which the lipid concentration in the haemolymph changed by 1.7 ± 2.3 mg/ml (n=5).

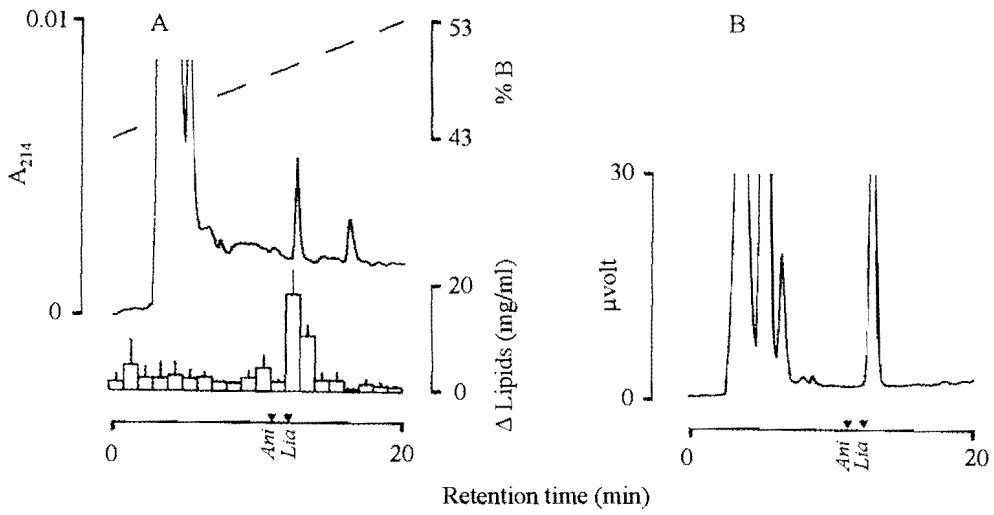


Fig. 4. RP-HPLC separation of CC from *P. inconspicuum* and *I. senegalensis*

UV absorbance at 214 nm was measured (A), and fluorescence in (B). The solvent system was as described in the legend to Fig. 2. The histogram below the chromatogram shows the adipokinetic activity upon injection of 2.5 CC equivalents of damselfly CC into acceptor locusts. The retention times of synthetic *Ani*-AKH and *Lia*-AKH are indicated.

4.3 Confirmation of sequences - HPLC and Mass Spectrometry

In order to supply confirmatory evidence that the novel peptides contain the assumed N-terminal pyroglutamic acid and C-terminal amide, the octapeptides *Ani*-AKH and *Psi*-AKH were synthesised. The synthetic and native peptides were compared using RP-HPLC. The novel synthetic *A. imperator* peptide (*Ani*-AKH) was eluted at essentially the same retention time as the active peak from a crude extract of *A. imperator* CC. Co-injection of active peak material and synthetic peptide resulted in co-elution on the various column materials (Table IX). The novel synthetic damselfly peptide (*Psi*-AKH) was similarly eluted at essentially the same retention time on various chromatographic supports as the active material isolated from a crude extract of *P. inconspicuum* CC (Table IX). A peptide with a free carboxyl terminus would have a different retention time to that of a peptide with an amidated C-terminus, as established previously on RP-HPLC for other AKH/RPCH family members (Hayes *et al.*, 1986; Gäde, 1990b);

Table IX. Chromatographic behaviour of native and synthetic AKH peptides

		Retention time (min)		
Peptide	Column	Natural	Synthetic	Co-injected
Ani-AKH	Nova-Pak C18	11.5	11.3	11.5
	Nova-Pak C8	11.8	11.9	
	Nova-Pak phenyl	14.2	14.3	
Psi-AKH	Nova-Pak C18	11.1	11.0	11.1
	Nova-Pak C8	11.3	11.1	11.1
	Nova-Pak phenyl	13.4	13.5	13.1

AKH peptides were isolated from CC of *A. imperator* and *P. inconspicuum*. The retention times of the natives peptides were compared chromatographically with the synthetic peptides *Ani*-AKH and *Psi*-AKH, respectively. The dimensions of the columns were 3.9 x 150 mm; particle size 4 µm; pore size 60 Å. The solvent system was as described in the legend to Fig. 2, with a flow rate of 0.5 ml/min. The gradient ran from 35 to 45% solvent B in 20 min.

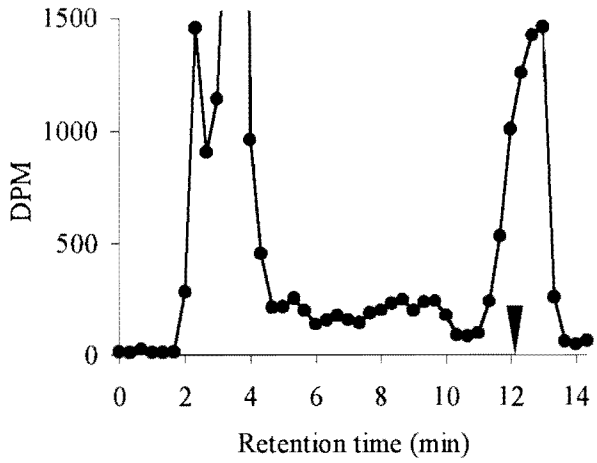
Further structural information was obtained by mass spectrometry. The mass of the intact peptide from *A. imperator* was measured using electrospray mass spectrometry as 946.4 Da. This mass represents the $[M+H]^+$ form of the peptide, and therefore the true mass of the peptide, is one mass unit less. The sequenced peptide, together with the N-terminal pGlu residue and C-terminal amide was calculated to have a molecular weight of 945 Da. These results provided additional evidence of the existence in this peptide of the C-terminal amide, and confirmed the sequence pGlu-Val-Asn-Phe-Ser-Pro-Ser-TrpNH$_2$

Mass spectrometry of the damselfly peptide was carried out using a MALDI spectrometer. The main ion was observed at m/z 952 using laser desorption mass spectrometric analysis. This mass was attributed to the $[M+Na]^+$ form of the peptide. The molecular mass of Na is 23 Da., and therefore the true measured mass was 929 Da. The above sequence plus the N-terminal pGlu residue and the C-terminal amide was calculated to have a molecular weight of 929 Da. These results identified the novel damselfly neuropeptide to have the sequence pGlu-Val-Asn-Phe-Thr-Pro-Gly-TrpNH$_2$.

4.4 Biological parameters

4.4.1 Site of synthesis of *Lia*-AKH in *O. julia*

Fig. 5. RP-HPLC separation of CC from *O. julia* incubated with ^{3}H-Phe



The separation was achieved using a Nova-Pak phenyl column using the same chromatographic conditions as in Table IX. 20 s. fractions were collected manually and radioactivity (expressed as DPM) measured by liquid scintillation counting. The retention time of *Lia*-AKH under the employed conditions is indicated.

Synthesis of neurosecretory compounds present in the CC may occur in the intrinsic glandular cells of the CC, or in neurosecretory cells of the brain. The neurosecretory products can then be transported by the nervi corporis cardiaci, and subsequently stored in the CC. Incubation of dissected CC with radioactively labelled ^{3}H-Phe can indicate whether *Lia*-AKH is stored, or synthesised in the CC of *O. julia*, and at the same time confirm the presence of Phe. The fractionation of 7 CC of *O. julia*, that had been incubated for 16 h with ^{3}H-Phe is shown in Fig. 5. A peak of radioactivity was eluted at an identical retention time (12.1 min) to synthetic *Lia*-AKH. The peak of radioactivity between 2 and 4 min represented the unincorporated ^{3}H-Phe.

4.4.2 Quantification per gland

The peak areas of known quantities of synthetic AKH/RPCH peptides during RP-HPLC fractionation on the Beckman System Gold were determined, and compared with the peak areas of the peptides purified from extracts of CC. Three aliquots of

glands of *P. inconspicuum* were calculated to contain 2.4 pmol *Psi*-AKH per gland. The same procedure, injecting known quantities of CC extracts from *A. imperator*, and calibrating the peak areas using known quantities of the synthetic peptide *Ani*-AKH, yielded 40 pmol per gland. Two extracts of CC prepared from *Aeshna minuscula* were found to have 30 and 35 pmol *Ani*-AKH per gland. One preparation of CC from a mixture of last instar larvae of *O. julia* and *C. erythraea*, had 8 pmol *Lia*-AKH per gland. Two extracts of CC prepared from teneral *O. julia* had 8 and 14 pmol *Lia*-AKH per gland. Two extracts of CC prepared from mature *O. julia* were found to have 19 and 24 pmol *Lia*-AKH per gland.

Table X. Bioactivity in Odonata of HPLC peaks and synthetic peptides

Acceptor insect:	Blood lipids (mg/ml)				Blood carbohydrates (mg/ml)			
Treatment	n	Before	After	Difference	n	Before	After	Difference
O. julia								
Control; water	5	31.9 ± 14.1	34.8 ± 15.8	2.9 ± 2.1	10	4.1 ± 2.3	4.4 ± 3.4	0.3 ± 1.9
Active HPLC fraction	5	23.2 ± 5.6	51.0 ± 10.4	27.8 ± 8.7**	5	6.4 ± 3.1	7.7 ± 3.7	1.3 ± 1.6
1 pmol *Lia*-AKH	10	27.6 ± 9.8	42.8 ± 12.7	15.0 ± 8.4***	11	6.9 ± 3.3	6.3 ± 2.4	-0.6 ± 2.1
A. imperator								
Control; water	7	55.3 ± 23.6	56.2 ± 22.1	0.9 ± 5.8	7	2.0 ± 1.0	2.6 ± 1.1	0.6 ± 0.5
Active HPLC fraction	6	56.4 ± 12.4	68.2 ± 12.3	11.8 ± 2.3***	6	1.7 ± 1.0	1.6 ± 1.1	-0.6 ± 0.7
3.4 pmol *Ani*-AKH	7	53.7 ± 19.1	61.5 ± 18.2	7.8 ± 4.8*	7	2.1 ± 1.3	2.8 ± 1.6	0.7 ± 1.3
P. inconspicuum								
Control; water	33		14.6 ± 4.3					
Active HPLC fraction	11		29.4 ± 9.6***					
Control; water	47		11.1 ± 4.3		36		4.8 ± 1.9	
1.0 pmol *Psi*-AKH	36		21.2 ± 8.2***					
2.5 pmol *Psi*-AKH	19		18.1 ± 7.0***		30		6.6 ± 3.2**	

Adults of the dragonflies, *O. julia* and *A. imperator* and the damselfly, *P. inconspicuum* were injected as indicated. The RP-HPLC purified fractions with adipokinetic activity in locusts (1.0 gland equivalent) were injected. Values are mean ± SD of the concentration of total lipids and carbohydrates in the haemolymph. The significance of the difference between values before and 90 min after injection is indicated by * $p<0.01$, ** $p<0.005$, and *** $p<0.001$. In the case of the damselfly the significance of differences between the control and experimental group is given.

4.4.3 Conspecific bioactivity of the Odonata AKH peptides

The effect of injection of the appropriate synthetic peptide on the concentration of haemolymph lipids and carbohydrates was assayed in *P. inconspicuum, O. julia* and

A. imperator (Table X). Injection of *O. julia* with 1 pmol *Lia*-AKH resulted in an increase of 54% of the initial concentration of lipids in the haemolymph. No significant effect on the concentration of carbohydrates in the haemolymph was measurable. Injection of *A. imperator* with 3.4 pmol *Ani*-AKH resulted in an increase of 14.5% of the initial concentration of lipids in the haemolymph. Injection with *Ani*-AKH had no measurable effect on the concentration of carbohydrates in the haemolymph. After injection of *P. inconspicuum* with 1 pmol *Psi*-AKH, the concentration of both lipids and carbohydrates in the haemolymph was increased significantly, by 91% and 38%, respectively (Table X).

4.4.4 Specificity of the adipokinetic response in *P. inconspicuum*

Pseudagrion inconspicuum were injected with 0.8 pmol of synthetic peptides, differing from the native *Psi*-AKH, in one (*Lia*-AKH), two (*Ani*-AKH) and three (*Pab*-RPCH) amino acid residues, and the lipid mobilising response measured. All peptides injected resulted in a significantly higher concentration of lipids in the haemolymph, compared with the control group (Table XI).

Table XI. Adipokinetic activity of various octapeptides in *P. inconspicuum*

Compound	n	Haemolymph lipid concentration (mg/ml)
Control; water	14	12.1 ± 5.7
pGlu-**Val**-Asn-Phe-**Thr**-Pro-**Gly**-Trp-NH_2 (*Psi*-AKH)	13	17.1 ± 8.6†
pGlu-**Val**-Asn-Phe-**Thr**-Pro-**Ser**-Trp-NH_2 (*Lia*-AKH)	11	18.0 ± 7.9†
pGlu-**Val**-Asn-Phe-**Ser**-Pro-**Ser**-Trp-NH_2 (*Ani*-AKH)	14	20.2 ± 9.6*
pGlu-**Leu**-Asn-Phe-**Ser**-Pro-**Gly**-Trp-NH_2 (*Pab*-RPCH)	8	22.8 ± 8.7**

Damselflies were injected with 0.8 pmol of the peptides. The values are mean ± SD of the concentration of lipids in the haemolymph 90 min after injection. The significance of the difference between the experimental groups and the water-injected control group is indicated by † $p<0.05$, * $p<0.01$ and ** $p<0.005$.

4.5 AKH/RPCH peptides of other Odonata

The AKH/RPCH peptides of the Odonata that have been found to date could be separated in a reproducible way from each other using RP-HPLC. This provides a reliable and powerful method for identification of AKH/RPCH peptides in extracts of CC from dragon- or damselflies, where sufficient material for sequencing cannot be obtained.

It was deemed worthwhile to investigate representatives of other families for the identity of their AKH peptides. When the sequences of the two novel dragonfly peptides, *Ani*-AKH and *Psi*-AKH, are compared with *Lia*-AKH and the crustacean *Pab*-RPCH (Table XI), it is clear that another, intermediary peptide may exist in nature. This unknown peptide, our "missing link" which may be called *Xxx*-AKH, has the same sequence as *Pab*-RPCH, with the exception that Val is substituted for Leu at position 2.

Adipokinetic material was identified by bioassay in locusts, after which the HPLC-purified peptides were N-terminally deblocked, and sequenced, when enough material was available. The sequences obtained are given in Table XIII (p. 55). The peptide identities based on (a) identification by retention time on RP-HPLC; (b) adipokinetic

activity in the bioassay, and (c) elucidation of the primary sequence are summarised in Table XIV (p. 56). The identity of an AKH/RPCH family peptide is considered established if two of the three criteria stated above have been met.

Chlorolestes fasciata (Zygoptera: Chlorolestidae)

A crude methanolic extract of *C. fasciata* CC was applied to a Nucleosil C18 column for RP-HPLC. A fraction was collected with both a UV and fluorescence peak, that had essentially the same retention time (11.6 min) as *Psi*-AKH (11.7 min). The compound was eluted later than both synthetic *Ani*-AKH (10.2 min) and *Lia*-AKH (11.1 min). This material was deblocked with pyroglutamate aminopeptidase and subjected to Edman sequencing. The peptide was found to have the same amino acid sequence as *Psi*-AKH (Table XIII). This result also confirmed the first evidence of the existence of *Psi*-AKH obtained with 3 CC of *C. tessellata* (Fig. 3 C & D).

Lestes tridens (Zygoptera: Lestidae)

A crude methanolic extract of *L. tridens* CC was applied to an Alphasil C18 column for RP-HPLC, and a fraction identified that had adipokinetic activity in locusts. Injection of the fraction (ca. 10 pmol peptide per locust) significantly increased the concentration of lipids in the haemolymph by 15.9 ± 4.4 mg/ml (n=6) ($p<0.001$), compared with a control group which showed no change (2.4 ± 2.0 mg/ml; n=6). The separation achieved on this column between *Lia*-AKH and *Psi*-AKH was not good. The retention time of *Psi*-AKH purified from *C. fasciata* was 16.56 min, and 16.47 min for synthetic *Lia*-AKH (Table XII). Despite this it was clear that the retention time of the active peptide from *L. tridens* is more similar to that of *C. fasciata*, than to the retention time of *Lia*-AKH and to the compound from the mixture of gomphids (which very likely was also *Lia*-AKH). It was therefore concluded that this peptide was *Psi*-AKH.

Table XII. RP-HPLC fractionation of CC extracts from *Lestes tridens* and *Chlorolestes fasciata*

	Native	Deblocked
Lestes tridens	16.53	14.08
Chlorolestes fasciata	16.56	14.13
Various gomphids[a]	16.44	13.92
Lia-AKH	16.47	13.95

[a] *Paragomphus cognatus, P. genei, Lestinogomphus angustus.* The RP-HPLC separation was achieved on an Alphasil C18 column using the same chromatographic conditions as in Fig. 2.

Lestes plagiatus (Zygoptera: Lestidae)

A fraction, with both a UV and fluorescence peak during RP-HPLC purification on a Nucleosil C18 column was found to have adipokinetic activity when tested in the locust bioassay, where it significantly increased haemolymph lipids by 15.9 ± 6.2 mg/ml (n=6) ($p<0.002$), compared with the increase in the control group (2.5 ± 2.8 mg/ml; n=6). The compound had an identical retention time (11.9 min) to *Psi*-AKH (11.9 min), and was eluted later than *Ani*-AKH (10.3 min) and *Lia*-AKH (11.3 min). This established that the compound was *Psi*-AKH.

Phaon iridipennis (Zygoptera: Calopterygidae)

A fraction, with both a UV and fluorescence peak during RP-HPLC purification on a Nucleosil C18 column, had an identical retention time (11.7 min) to *Psi*-AKH (11.7 min) and was eluted later than *Ani*-AKH (10.2 min) and *Lia*-AKH (11.1 min). Too little material was present to show activity in the locust bioassay (ca. 1.5 pmol of the peptide per locust were injected, not taking any losses into account). It can therefore only tentatively be assumed that this compound could be *Psi*-AKH.

Chlorocypha caligata (Zygoptera: Chlorocyphidae)

A fraction, with both a UV and fluorescence peak during RP-HPLC purification on a Nucleosil C18 column, had an identical retention time (11.9 min) to *Psi*-AKH

(11.9 min) and was eluted later than *Ani*-AKH (10.3 min) and *Lia*-AKH (11.3 min). The fraction also had adipokinetic activity in the locust bioassay (ca. 12.5 pmol were injected per locust), significantly increasing lipids in the haemolymph by 23.1 ± 11.9 mg/ml (n=6) ($p<0.005$), compared with a control group where the change in haemolymph lipids was 2.5 ± 3.3 mg/ml (n=6). This compound was therefore identified as *Psi*-AKH.

Allocnemis leucosticta (Zygoptera: Protoneuridae)

A fraction, with both a UV and fluorescence peak during RP-HPLC purification on a Nucleosil C18 column, had an identical retention time (11.95 min) to *Psi*-AKH (11.9 min) and was eluted later than *Ani*-AKH (10.3 min) and *Lia*-AKH (11.3 min).

Ictinogomphus ferox (Anisoptera: Gomphidae)

A fraction, with both a UV and fluorescence peak during RP-HPLC purification on a Nucleosil C18 column, had essentially the same retention time (11.4 min) as *Lia*-AKH (11.3 min) and was eluted later than *Ani*-AKH (10.3 min) and earlier than *Psi-AKH* (11.9 min). The fraction also had adipokinetic activity in the locust bioassay (ca. 6 pmol were injected per locust), significantly increasing lipids in the haemolymph by 17.5 ± 7.0 mg/ml (n=6) ($p<0.002$), compared with a control group where the change in haemolymph lipids was 2.5 ± 3.3 mg/ml (n=6). Sufficient material was deblocked, RP-HPLC purified and subjected to Edman sequencing. The peptide was identified as *Lia*-AKH based on its amino acid sequence (Table XIII), confirming the initial results based on the retention time of the bioactive compound.

Ceratogomphus pictus (Anisoptera: Gomphidae)

A fraction, with both a UV and fluorescence peak during RP-HPLC purification on a Nucleosil C18 column, had an identical retention time (11.0 min) to *Lia*-AKH (11.0 min) and was eluted later than *Ani*-AKH (9.9 min) and earlier than *Psi*-AKH (11.3 min). The fraction also had adipokinetic activity in the locust bioassay significantly increasing the lipids in the haemolymph by 16.9 ± 5.7 mg/ml (n=5) ($p<0.005$) (Table VII). Sufficient material was deblocked, RP-HPLC purified and

subjected to Edman sequencing. The peptide's amino acid sequence (Table XIII), confirmed that the identified bioactive compound was *Lia*-AKH.

Anotogaster sieboldii (Anisoptera: Cordulegasteridae)

A fraction, with both a UV and fluorescence peak during RP-HPLC purification on a Waters Nova-Pak phenyl column, had an identical retention time (10.0 min) to *Ani*-AKH (10.0 min) and was eluted earlier than *Lia*-AKH (11.4 min). During RP-HPLC purification on a Nucleosil C18 column the same compound had a retention time of 10.8 min, and was eluted earlier than *Lia*-AKH (12.1 min) and *Psi*-AKH (12.6 min). The fraction had adipokinetic activity in the locust bioassay (ca. 9 pmol peptide were injected per locust) significantly increasing the lipids in the haemolymph by 15.7 ± 7.5 mg/ml (n=6) ($p<0.005$) compared with the control group which showed a change of lipids in the haemolymph of 1.2 ± 1.3 mg/ml (n=6). Enough material was deblocked using pyroglutamate aminopeptidase, purified by RP-HPLC and subjected to Edman sequencing. The peptide's amino acid sequence (Table XIII), confirmed that the identified bioactive compound was *Ani*-AKH.

Aeshna minuscula (Anisoptera: Aeshnidae)

A fraction, with both a UV and fluorescence peak during RP-HPLC purification on a Nucleosil C18 column, had an identical retention time (10.2 min) to *Ani*-AKH (10.2 min) and was eluted earlier than *Lia*-AKH (11.1 min) and *Psi*-AKH (11.7 min). The lack of bioassay data means that this compound can only tentatively be identified as *Ani*-AKH.

Aeshna subpupillata (Anisoptera: Aeshnidae)

A fraction, with both a UV and fluorescence peak during RP-HPLC purification on a Nucleosil C18 column (chromatographic conditions were as described in the legend to Table V), had an identical retention time (18.7 min) to *Ani*-AKH (18.7 min) and was eluted earlier than *Lia*-AKH (20.5 min). This fraction also had adipokinetic activity when tested in the locust bioassay (ca. 5.5 pmol peptide injected per locust), significantly increasing lipids in the haemolymph by 21.0 ± 9.3 mg/ml (n=5) ($p<0.01$)

compared with the control group where the lipids changed by 2.4 ± 2.0 mg/ml (n=6). Enough material was deblocked using pyroglutamate aminopeptidase, purified by RP-HPLC and subjected to Edman sequencing. The peptide was identified as *Ani*-AKH based on its amino acid sequence (Table XIII), confirming the initial results.

Syncordulia gracilis (Anisoptera: Corduliidae)

A fraction, with both a UV and fluorescence peak during RP-HPLC purification on a Nucleosil C18 column, had essentially the same retention time (11.0 min) as *Ani*-AKH (10.9 min) and was eluted earlier than *Lia*-AKH (12.5 min). Only ca. 3 pmol peptide (assuming no losses) of the purified fraction were available for injection per locust which was too little to establish bioactivity. The lack of bioassay data means that this compound can only tentatively be identified as *Ani*-AKH.

Crocothemis erythraea (Anisoptera: Libellulidae)

A fraction, with both a UV and fluorescence peak during RP-HPLC purification on a Nucleosil C18 column, had an identical retention time (13.3 min) to *Lia*-AKH (13.3 min) and was eluted later than *Ani*-AKH (12.1 min) and earlier than *Psi*-AKH (14.2 min). This fraction also had biological activity in the locust bioassay (Table VI). Sufficient material was deblocked using pyroglutamate aminopeptidase and subjected to Edman sequencing. The peptide sequence established that this peptide was *Lia*-AKH (Table XIII), confirming the conclusion based on retention time and bioactivity.

Bradinopyga cornuta (Anisoptera: Libellulidae)

A fraction, with both a UV and fluorescence peak during RP-HPLC purification on a Nucleosil C18 column, had an identical retention time (8.8 min) to *Lia*-AKH (8.8 min) and was eluted later than *Ani*-AKH (8.0 min) and earlier than *Psi*-AKH (9.1 min). The AKH peptides were eluted earlier than normal, as an error had been made during the preparation of the solvents. The percentage of the organic modifier (ACN) in solvent B was too high. Too little material was available to show activity in

the locust bioassay. The compound can therefore only tentatively be identified as *Lia*-AKH.

Sympetrum vicinum (Anisoptera: Libellulidae)

A fraction, with both a UV and fluorescence peak during RP-HPLC purification on a Nucleosil C18 column, had essentially the same retention time (13.4 min) as *Lia-AKH* (13.3 min) and was eluted later than *Ani*-AKH (12.4 min) and later than *Psi*-AKH (14.3 min). This fraction had activity in the locust bioassay (ca. 8.5 pmol were injected per locust), and significantly increased the lipid concentration in the haemolymph by 17.4 ± 3.9 mg/ml (n=6) ($p<0.001$) compared with the control group which had a change in lipids of 1.1 ± 1.9 mg/ml (n=6). The bioactive compound was therefore identified as *Lia*-AKH.

Sympetrum infuscatum (Anisoptera: Libellulidae)

A fraction, with both a UV and fluorescence peak during RP-HPLC purification on a Nucleosil C18 column, had essentially the same retention time (12.1 min) as *Lia-AKH* (12.0 min), was eluted later than *Ani*-AKH (10.8 min) and earlier than *Psi*-AKH (12.7 min). This fraction had adipokinetic activity in the locust bioassay (ca. 5 pmol peptide injected per locust), and significantly increased the lipid concentration in the haemolymph by 18.1 ± 8.9 mg/ml (n=6) ($p<0.005$) compared with the control group which had a change in lipids of 1.7 ± 2.6 mg/ml (n=6). The bioactive compound was therefore identified as *Lia*-AKH.

Trithemis dorsalis and *T. stictica* (Anisoptera: Libellulidae)

A fraction, with both a UV and fluorescence peak during RP-HPLC purification on a Nucleosil C18 column, had an identical retention time (13.3 min) to *Lia-AKH* (13.3 min) and was eluted later than *Ani*-AKH (12.1 min) and earlier than *Psi*-AKH (14.3 min). This fraction, from each of the species individually, had adipokinetic activity in the locust bioassay (Table VI). Material from both species was therefore combined to provide enough material for structural elucidation, deblocked using

pyroglutamate aminopeptidase, and subjected to Edman sequencing. The amino acid sequencing results confirmed that this peptide was *Lia*-AKH (Table XIII).

Trithemis arteriosa (Anisoptera: Libellulidae)

A fraction, with both a UV and fluorescence peak during RP-HPLC purification on a Nucleosil C18 column, had essentially the same retention time (11.2 min) as *Lia-AKH* (11.1 min) and was eluted later than *Ani*-AKH (10.2 min) and earlier than *Psi*-AKH (11.7 min). Material of this fraction was deblocked using pyroglutamate aminopeptidase, and subjected to Edman sequencing. The sequencing results proved that this peptide was *Lia*-AKH (Table XIII).

Pantala flavescens (Anisoptera: Libellulidae)

A fraction, with both a UV and fluorescence peak during RP-HPLC purification on a Nucleosil C18 column, had a similar retention time (12.2 min) to *Lia*-AKH (12.1 min). This fraction had adipokinetic activity when injected into locusts (Table VI p. 37). Material of this fraction was deblocked using pyroglutamate aminopeptidase, and subjected to Edman sequencing. The sequencing results confirmed that this peptide was *Lia*-AKH (Table XIII).

Brachythemis leucosticta (Anisoptera: Libellulidae)

A fraction, with both a UV and fluorescence peak during RP-HPLC purification on a Nucleosil C18 column had essentially the same retention time (11.2 min) as *Lia*-AKH (11.1 min) and was eluted later than *Ani*-AKH (10.2 min) and earlier than *Psi*-AKH (11.7 min). This fraction had adipokinetic activity when ca. 5 pmol peptide were injected into locusts, significantly increasing the concentration of lipids in the haemolymph by 12.1 ± 4.8 mg/ml (n=6) ($p<0.002$). Material of this fraction was deblocked using pyroglutamate aminopeptidase, and subjected to Edman sequencing. The amino acid sequence obtained was the same as *Lia*-AKH (Table XIII).

Table XIII. Amino acid sequences of Odonata adipokinetic peptides

	Edman cycle							
	1	2	3	4	5	6	7	8
Pseudagrion inconspicuum *Ischnura senegalensis*	V (34)	N (18)	F (17)	T (13)	P (10)	G (15)	W (3)	-
Chlorolestes fasciata	V	N	F	T	P	G	W	-
Anax imperator	V (140)	N (67)	F (84)	S (44)	P (49)	S (27)	W (7)	-
Anax speratus	V (96)	N (48)	F (65)	S (34)	P (43)	S (19)	W (3)	-
Aeshna subpupillatum	V (99)	N (58)	F (81)	S (34)	P (49)	S (29)	W (18)	-
Ictinogomphus ferox	V (53)	N (28)	F (49)	T (22)	P (18)	S (6)	W (+)	-
Ceratogomphus pictus	V (45)	N (19)	F (40)	T (23)	P (9)	S (11)	W (5)	-
Anotogaster sieboldii	V	N	F	S	P	S	W	-
Trithemis dorsalis *Trithemis stictica* *Orthetrum julia falsum*	V (90)	N (48)	F (61)	T (50)	P (41)	S (22)	W (6)	-
Orthetrum julia falsum	V (60)	N (29)	F (41)	T (27)	P (33)	S (13)	W (10)	-
Crocothemis erythraea	V (32)	N (14)	F (21)	T (13)	P (14)	S (4)	W (3)	-
Brachythemis leucosticta	V (16)	N (10)	F (13)	T (9)	P (8)	S (5)	W (1)	-
Trithemis arteriosa	V (13)	N (8)	F (9)	T (8)	P (6)	S (4)	W (1)	-
Pantala flavescens	V (88)	N (50)	F (99)	T (59)	P (20)	S (7)	W (3)	-

RP-HPLC purified peptides were deblocked using pyroglutamate aminopeptidase, purified by RP-HPLC, and subjected to automated Edman sequencing. The one letter abbreviation for the amino acid determined at each position, and in brackets, the yield in pmol, are given.

Table XIV. The AKH/RPCH peptides of the Odonata

	Peptide	Method of determination		
Zygoptera				
Chlorolestidae				
Chlorolestes fasciata	*Psi*-AKH	HPLC	-	Sequenced
Chlorolestes tesselata	*Psi*-AKH	HPLC	Bioassay	-
Lestidae				
Lestes tridens	*Psi*-AKH	HPLC	Bioassay	-
Lestes plagiatus	*Psi*-AKH	HPLC	Bioassay	-
Calopterygidae				
Phaon iridipennis	*Psi*-AKH ?	HPLC	-	-
Chlorocyphidae				
Chlorocypha caligata	*Psi*-AKH	HPLC	Bioassay	-
Protoneuridae				
Allocnemis leucosticta	*Psi*-AKH ?	HPLC	-	-
Coenagrionidae				
Pseudagrion inconspicuum	*Psi*-AKH	HPLC	Bioassay	Sequenced
Ischnura senegalensis	*Psi*-AKH	HPLC	Bioassay	Sequenced
Anisoptera				
Aeshnidae				
Anax imperator	*Ani*-AKH	HPLC	Bioassay	Sequenced
Anax speratus	*Ani*-AKH	HPLC	Bioassay	Sequenced
Aeshna miniscula	*Ani*-AKH ?	HPLC	-	-
Aeshna subpupillata	*Ani*-AKH	HPLC	Bioassay	Sequenced
Gomphidae				
Ceratogomphus pictus	*Lia*-AKH	HPLC	Bioassay	Sequenced
Ictinogomphus ferox	*Lia*-AKH	HPLC	Bioassay	Sequenced
Cordulegasteridae				
Anotogaster sieboldii	*Ani*-AKH	HPLC	Bioassay	Sequenced
Corduliidae				
Syncordulia gracilis	*Ani*-AKH ?	HPLC	-	-
Libellulidae				
Crocothemis erythraea	*Lia*-AKH	HPLC	Bioassay	Sequenced
Bradinopyga cornuta	*Lia*-AKH ?	HPLC	-	-
Sympetrum vicinum	*Lia*-AKH	HPLC	Bioassay	-
Sympetrum infuscatum	*Lia*-AKH	HPLC	Bioassay	-
Trithemis dorsalis	*Lia*-AKH	HPLC	Bioassay	Sequenced
Trithemis stictica	*Lia*-AKH	HPLC	-	Sequenced
Trithemis arteriosa	*Lia*-AKH	HPLC	-	Sequenced
Pantala flavescens	*Lia*-AKH	HPLC	Bioassay	Sequenced
Brachythemis leucosticta	*Lia*-AKH	HPLC	Bioassay	Sequenced

The identity of the AKH/RPCH peptide of a species was considered established if two or more criteria (identical retention time on RP-HPLC to a synthetic peptide, biological activity, or sequence) had been met. A question mark indicates that only one of the criteria had been determined.

4.6 Morphological measurements

Table XV summarises the fresh weights of whole animal, as well as flight muscles, and haemolymph volume of *O. julia*, *A. imperator*, *P. inconspicuum* and *I. senegalensis*.

Table XV. Morphological measurements

	Whole animal: fresh weight (mg)		Flight muscles: fresh weight (mg)		Haemolymph volume (µl)	
O. julia	256.0 ± 36.0	29[a]	56.2 ± 13.5	13[a]	30.5 ± 9.1	8[a]
A. imperator	1154.0 ± 115.0	18[b]	113.9 ± 40.9	16		
	909.0 ± 73.0	20[a]				
P. inconspicuum	43.5 ± 3.5	21[a]			6.5 ± 1.4	7[a]
					7.7 ± 1.8	5[b]
I. senegalensis	33.1 ± 3.4	14[a]			5.4 ± 2.5	9

Values are mean ± SD of fresh weight or haemolymph volume (determined by the ^{3}H-inulin dilution method) of adult dragon- and damselflies. The number of measured individuals, males (a), as well as females (b) are given. The fresh weight of the flight muscles of *A. imperator* was determined from males and females. The haemolymph volume of *I. senegalensis* was determined from 6 males and 3 females.

The fresh weight of flight muscles as a percentage of the fresh weight of the whole animal is ca. 22% in *O. julia*, and 11% in *A. imperator*.

4.7 Parameters of energy metabolism

4.7.1 Enzyme activities

The activities of the enzymes HOAD, GAPDH, PK, CS, PFK and LDH in flight muscles of *O. julia* and *A. imperator* were compared with that of *L. migratoria*.

Table XVI. Enzyme activities in flight muscles

	Glycogen phosphorylase	PFK	GAPDH	HOAD	CS
L. migratoria	9.3 ± 0.5 (4)	17.4 ± 1.6 (4)	92 ± 33 (4)	27.4 ± 6.4 (10)	75.4 ± 5.8 (4)
O. julia	3.6 ± 1.0 (4)	27.9 ± 1.4 (5)	102 ± 8 (4)	16.9 ± 5.8 (16)	75.2 ± 15.3 (5)
A. imperator	2.8 ± 0.6 (4)	18.7 ± 5.3 (4)	100 ± 21 (3)	22.0 ± 7.6 (10)	99.0 ± 22.1 (5)

Values are mean ± SD of the maximal enzyme activities in the flight muscles, expressed as U/g, with the number of independent samples in brackets.

All enzyme activities are expressed as mean ± SD of U/g fresh weight of muscle (Table XVI). The maximal activity of the glycogen phosphorylase a+b in the flight muscles of *L. migratoria* was found to be between 2.5- and 3-fold higher than in the dragonflies, *O. julia* and *A. imperator* ($\alpha<0.001$). The maximal activity of PFK in flight muscle extracts from *O. julia* was found to be 1.5-fold the activity measured in *A. imperator* or *L. migratoria* ($\alpha<0.001$). The maximal activity of another glycolytic enzyme, PK, was much higher than PFK, and measured as 111 ± 27 (*A. imperator*), 108 ± 22 (*O. julia*), and 144 ± 23 (*L. migratoria*) (n=4). The activity of LDH in flight muscles was very low, and ranged from 0.5 ± 0.4 (*A. imperator*), to 0.4 ± 0.05 (*O. julia*), and 1.1 ± 0.5 (*L. migratoria*) (n=4). The femur muscle of *L. migratoria* is known to contain high activities of LDH (53.0 U/g; Crabtree and Newsholme, 1972). A control enzyme assay was therefore conducted on two extracts of femur muscle and the LDH activity was measured as 56.6 and 110.2 U/g.

The maximal activity of HOAD in flight muscles of *L. migratoria* was 1.5-fold higher than in *O. julia* ($\alpha<0.001$). The differences in the maximal activity of HOAD in the flight muscles when comparing *A. imperator* and *O. julia*, or *A. imperator* and *L. migratoria* were not statistically significant. No major differences were measured in the activity of GAPDH in the flight muscles of *L. migratoria, O. julia* and *A. imperator*. *Anax imperator* seems to have a higher CS activity in flight muscles than *O. julia* and *L. migratoria*, but this difference is not statistically significant. The values are in the same order of magnitude as measured for GAPDH.

4.7.2 Rates of oxidation of substrates by mitochondria

Cytox activity in whole flight muscle homogenates is expressed as mean ± SD of μmol O_2 consumed/g/min. The maximal activity of Cytox measured in *A. imperator* was 90.5 ± 30.8 (n=4), in *O. julia* 89.1 ± 26.1 (n=8) and in *L. migratoria* 57.1 ± 13.7 (n=3). The rates of O_2 consumption of the substrates added to the isolated mitochondria were expressed as a percentage of the maximal Cytox activity determined separately for each mitochondrial preparation.

α-Glycerol-phosphate was oxidised at the highest rate of all substrates by the mitochondria isolated from flight muscles, and the rate of oxidation of this substrate was similar in *L. migratoria, O. julia* as well as in *A. imperator* (Table XVII). Pyruvate was found to be oxidised at similar rates by *O. julia* and *L. migratoria.* Only two determinations were made for pyruvate oxidation by mitochondria of *A. imperator*, but it seems unlikely that there are major differences from *L. migratoria* or *O. julia.* In *A. imperator* palmitoyl-carnitine was oxidised at 1.5-fold the rate of *L. migratoria* or *O. julia.* The difference in the mean values is not statistically significant, due to the high standard deviation, and low number of replicates performed.

Table XVII. Substrate oxidation by mitochondria isolated from flight muscles

Substrate	*L. migratoria*		*O. julia*		*A. imperator*	
α–Glycerolphosphate	13.20 ± 2.80	(5)	11.92 ± 2.95	(5)	9.60 ± 1.21	(3)
Pyruvate	2.35 ± 0.84	(6)	1.82 ± 0.48	(5)	2.84	(2)
Malate	1.25 ± 0.03	(3)	3.44 ± 1.28	(4)	4.89 ± 1.12	(3)
Succinate	2.98 ± 0.56	(3)	4.70 ± 2.05	(5)	3.56 ± 0.42	(3)
Proline	2.16 ± 0.61	(7)	2.75 ± 0.84	(6)	3.32 ± 0.56	(4)
Palmitoyl-carnitine	2.30 ± 0.77	(5)	2.34 ± 1.01	(5)	3.31 ± 0.88	(3)

Values are mean ± SD of oxygen consumption rates of substrates by mitochondrial preparations from flight muscles of locust and dragonflies. Values in brackets are the number of independent samples (n). The rates are expressed as a percentage of the activity of Cytox.

The rate of oxidation of Pro in *A. imperator* was 1.5-fold higher than in *L. migratoria*. The difference was statistically significant only at the level of $\alpha<0.05$. There was no significant difference between the rates of oxidation of Pro measured in *O. julia* and *L. migratoria* or *A. imperator*. The rate of oxidation of malate in *A. imperator* was 4-fold higher than in *L. migratoria*. The difference was statistically significant at a level of $\alpha<0.01$. There was no difference between the rates of oxidation of malate measured in *O. julia* and *L. migratoria* or *A. imperator*, as a result of the high standard deviation, and small sample size. Succinate was oxidised at similar rates in *L. migratoria, A. imperator* and *O. julia*. None of the means was significantly different from any other.

4.7.3 Metabolites in the haemolymph

The resting lipid and carbohydrate concentration in the haemolymph of adults and larvae of *A. imperator*, adults of *O. julia*, larvae of a mixture of *O. julia* and *C. erythraea*, and adult *P. inconspicuum* were compared (Fig. 6). The significance of the differences between any two groups is summarised in Table XVIII.

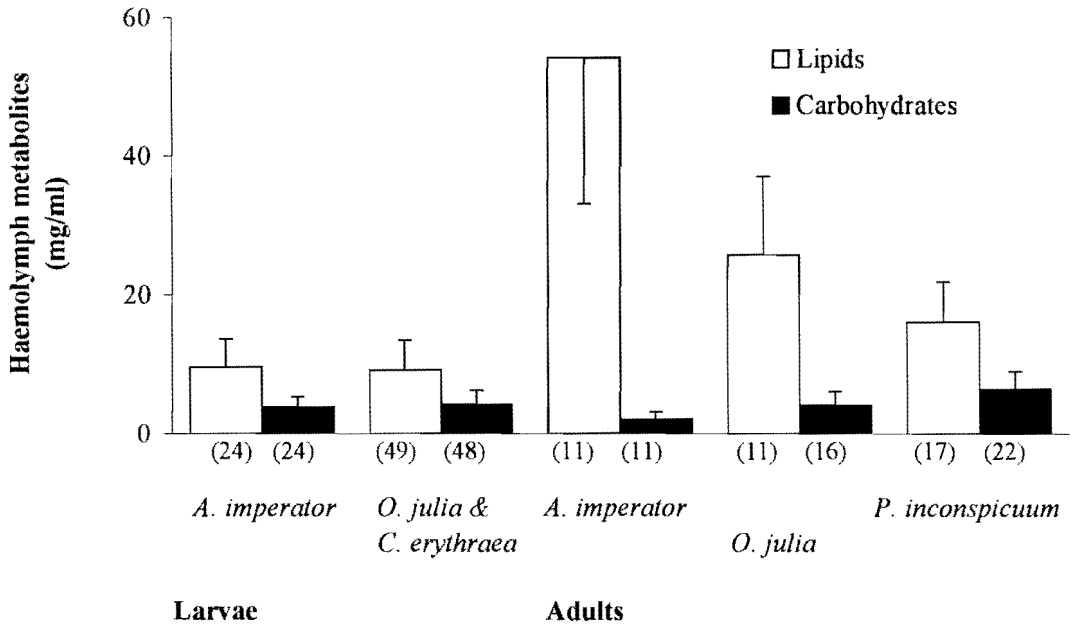


Fig. 6. Comparison of lipid and carbohydrate concentrations in the haemolymph

Bars represent means, error bars are SD of the resting haemolymph concentration of lipids and carbohydrates in the haemolymph, and the number of samples are given in brackets.

In the adults the measured lipid concentration in the haemolymph was the highest in *A. imperator*. The lipid concentration in *O. julia* was about half of this, and the lowest concentration was measured in *P. inconspicuum*. The highest concentration of carbohydrates in the haemolymph was measured in *P. inconspicuum* and the lowest in *A. imperator*. The ratio of lipid to carbohydrate in the haemolymph ranged from 27:1 in *A. imperator*, to 4:1 in O. julia and 2.5:1 in *P. inconspicuum*. In the larvae there was no difference in the lipid and carbohydrate concentration when *C. erythraea* and *O. julia* were compared with *A. imperator*. The ratio of lipid to carbohydrate was ca. 2:1 in both cases. *Anax imperator* adults have a carbohydrate concentration that is about half that found in the larvae, but conversely the lipid concentration is 5-fold higher. Adult *O. julia* have a similar carbohydrate concentration to that of their larvae, but the lipids in the haemolymph are found at two to three times the concentration of that of the larvae.

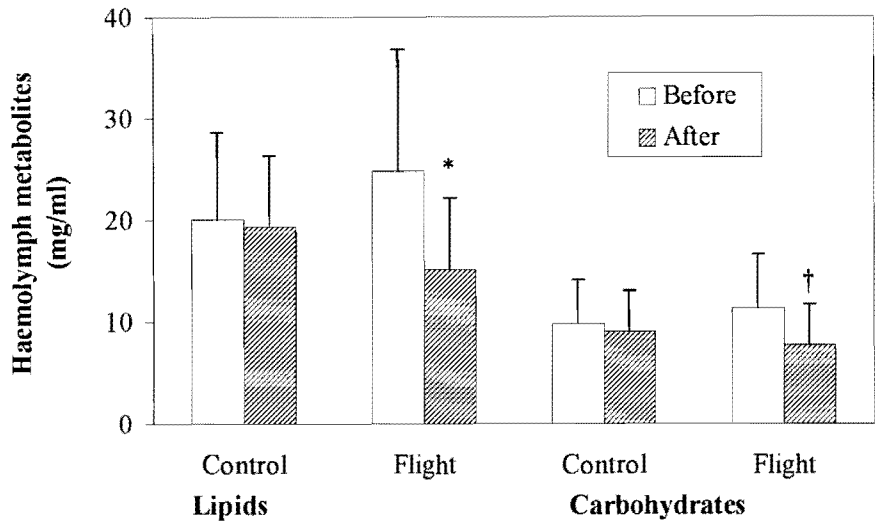
Table XVIII. Significance of differences amongst resting concentrations of lipids and carbohydrates in the haemolymph of Odonata

			Lipids						Carbohydrates					
			Larvae		Adults				Larvae		Adults			
			A. imperator	*O. julia* & *C. erythraea*	*A. imperator*	*O. julia*	*P. inconspicuum*		*A. imperator*	*O. julia* & *C. erythraea*	*A. imperator*	*O. julia*	*P. inconspicuum*	
Lipids	Larvae	*A. imperator*	-	N.S.	***	***	‡		†	*	‡	N.S.	N.S.	
		O. julia & *C. erythraea*	N.S.	-	***	***	**		‡	**	‡	N.S.	N.S.	
	Adults	*A. imperator*	***	***	-	***	***		***	***	***	***	***	
		O. julia	***	***	***	-	**		***	***	***	***	***	
		P. inconspicuum	‡	**	***	**	-		***	***	***	***	***	
Carbohydrates	Larvae	*A. imperator*	†	‡	***	***	***		-	N.S.	N.S.	N.S.	N.S.	
		O. julia & *C. erythraea*	*	**	***	***	***		N.S.	-	N.S.	N.S.	N.S.	
	Adults	*A. imperator*	‡	‡	***	***	***		N.S.	N.S.	-	N.S.	N.S.	
		O. julia	N.S.	N.S.	***	***	***		N.S.	N.S.	N.S.	-	N.S.	
		P. inconspicuum	N.S.	N.S.	***	***	***		N.S.	N.S.	N.S.	N.S.	-	

The significance of differences between the mean lipid or carbohydrate concentration of adults, and larvae of Odonata is indicted by † $\alpha<0.05$, ‡ $\alpha<0.025$, * $\alpha<0.01$, ** $\alpha<0.005$, *** $\alpha<0.001$ and was calculated using ANOVA and the Tukey test. N.S. indicates that the difference between the values was not statistically significant.

During a flight lasting 30 min, the concentration of total lipids in the haemolymph of *O. julia* was decreased significantly ($p<0.01$) by 9.7 ± 7.1 mg/ml (n=12), compared with the control group where the change was -0.7 ± 3.1 mg/ml (n=9). The concentration of total carbohydrates in the haemolymph was reduced significantly ($p<0.05$) by 3.6 ± 5.1 mg/ml, compared with the control group where the change was -0.8 ± 2.2 mg/ml (Fig. 7).

Fig. 7. Flight and the concentration of lipids and carbohydrates in the haemolymph of *O. julia*



The significance of the difference between values before and after 30 min of flight is indicated by † $p<0.05$ and * $p<0.01$.

The change in concentration of the free amino acids Pro and Ala in the haemolymph was also investigated during flight in *O. julia*. Proline was found to decrease by 64% during 30 min of flight (Table XIX). No change in the concentration of Pro in the haemolymph of the control group was detected. There was no significant change in the concentration of Ala in the haemolymph during flight, and no change was measured in the control group.

Table XIX. Flight and the concentration of Pro and Ala in the haemolymph of *O. julia*

Treatment		Haemolymph proline (mM)			Haemolymph alanine (mM)		
	n	Before	After	Difference	Before	After	Difference
Control; no flight	6	12.4 ± 2.9	12.2 ± 2.5	-0.2 ± 1.0	0.4 ± 0.2	0.3 ± 0.1	-0.1 ± 0.2
30 min flight	9	14.3 ± 1.8	5.2 ± 2.6	-9.1 ± 2.2***	0.4 ± 0.1	0.7 ± 0.4	0.3 ± 0.4

Values are mean ± SD of the concentration in the haemolymph of Ala and Pro. The significance of the difference between the control and experimental group is indicated by *** $p<0.001$.

4.7.4 Metabolite content in the flight muscles

In insects glucose derived from the diet is converted either into glycogen or trehalose (Steele, 1981). Glycogen is the chief reserve form of carbohydrate in insects (Wyatt, 1967). The carbohydrate content of the flight muscles (principally glycogen) of a group of *O. julia* that flew for 30 min was 0.14 ± 0.058 mg glucose equivalents/g (n=12). This amount differs significantly ($p<0.001$) from a control group where the glycogen content was measured to be 0.31 ± 0.10 mg glucose equivalents/g (n=10). Flight therefore reduced the initial carbohydrate content of the flight muscle by 54%.

Proline was determined in extracts of flight muscles of unflown *O. julia* to be 12.6 ± 1.8 (n=10) µmol/g, whereas the Pro content in the flight muscle of *O. julia* after flight was significantly lower ($p<0.001$) namely 3.1 ± 2.6 µmol/g (n=12). The Ala content in flight muscles of the control group were measured as 5.4 ± 1.4 (n=10) µmol/g, whereas the concentration in the flown group was significantly lower ($p<0.001$) (2.3 ± 0.9 µmol/g; n=12).

5. DISCUSSION

5.1 Biological activity in CC of Odonata

Once the presence of adipokinetic and hypertrehalosaemic compounds in the CC of Odonata other than *L. auripennis* was established (Table III; p. 30), the CC extracts were investigated for a potential adipokinetic or hypertrehalosaemic effect when injected into the native species. The increase in haemolymph lipids in the water-injected control group in the initial experiments performed on *A. imperator* may be attributed to the release of endogenous factors stimulating lipid mobilisation. These factors may include the adipokinetic hormone, but it could also be due to release of the catecholamine octopamine, initiating a "fight or flight" response. In locusts the stress of handling leads to a rapid increase in the titre of octopamine in the haemolymph which can lead to a small, and prolonged rise in the concentration of lipids in the haemolymph (Goldsworthy, 1990).

The maximum response of locusts to injection of conspecific CC extract varied from one batch of locusts to the next, despite the homogeneous conditions under which they were kept. The differences in the increase in the concentration of lipids in the haemolymph (Table III; p. 30) are attributable to age and/or physiological state of the animals. When performing physiological experiments on dragonflies that have been collected from the wild, very little can be done to compensate for differences in physiological state, or age. This is probably responsible for the high standard deviation of the mean values of most measurements in the Odonata.

The adipokinetic response resulting from injection of adult *O. julia, A. imperator* and *P. inconspicuum* with an extract from conspecific CC is evidence for the storage of an adipokinetic factor in this gland, that could be responsible for regulation of lipid homeostasis. No hyperglycaemic effect in the haemolymph could be demonstrated in either dragonfly. In contrast, CC extract had a small, but highly significant hyperglycaemic effect in *P. inconspicuum.* Considering the effect of a temperature of 10°C on the magnitude of the adipokinetic effect in locusts, it is possible that the increases reported in the concentration of lipids in the haemolymph of *A. imperator* and *O. julia* may not be the maximal possible.

In similar experiments on larvae of a mixture of *O. julia* and *C. erythraea* as well as *A. imperator*, no hyperglycaemic or adipokinetic effect could be demonstrated. This is in contrast to the clear adipokinetic effect of CC in dragonfly larvae as demonstrated by Tembhare and Andrew (1991). These larvae were fed *ad libitum* in the laboratory for some weeks prior to the experiments being performed. The lack of an adipokinetic or hyperglycaemic response in dragonfly larvae in this study could be a result of starvation. If all reserves were depleted no lipids, or carbohydrates could be mobilised. In *L. migratoria* starvation depletes the carbohydrate reserves of the fat body and the haemolymph, and starved adult males do not exhibit a hyperglycaemic response to injection with CC (Goldsworthy, 1969). Another explanation is the possibility that the larvae used in this study were of a different age compared with the larvae used by Tembhare and Andrew (1991). It is well known that in locusts the magnitude of the adipokinetic effect after injection of adipokinetic hormone is age-dependent (Mwangi and Goldsworthy, 1977).

Elucidation of the primary structure of these adipokinetic factors confirmed the earlier results based on the retention time on HPLC during purification. Two novel peptides were discovered: one isolated from CC of *A. imperator* (*Ani*-AKH), and the other from *P. inconspicuum* and *I. senegalensis* (*Psi*-AKH). The power of RP-HPLC to correctly identify AKH peptides based on retention time is shown by the clear separation of *Psi*-AKH from *Ani*-AKH or *Lia*-AKH (Fig. 4 p. 42). The peptides are typical members of the AKH/RPCH family of peptides because they possess the following characteristics:

1. they are uncharged;
2. the N-terminus of both peptides is blocked by a pyroglutamyl residue, verified by enzymatic cleavage and mass data;
3. the C-terminus is blocked by an amide;
4. Phe and Trp residues at positions 4 and 8, respectively.

Many neuropeptides are amidated at the C-terminus, effectively blocking the free carboxyl group, probably for protection against rapid unspecific degradation by carboxypeptidases in the blood (Goldsworthy and Mordue, 1989). Evidence for the presence of this C-terminal structural modification in *Lia*-AKH isolated from

L. auripennis was provided by the fact that the peptide had not been degraded after incubation with carboxypeptidase A (Gäde, 1990a). Comparing the mass data obtained for native *Ani*-AKH and *Psi*-AKH with calculated theoretical masses, also provides evidence for the existence of a C-terminal amide. More proof that the primary structures had been correctly elucidated was provided by biological activity exerted upon injection of the respective synthetic compounds into their species of origin. Injecting synthetic *Lia*-AKH into *O. julia*, *Ani*-AKH into *A. imperator*, and *Psi*-AKH into *P. inconspicuum*, resulted in a highly significant adipokinetic effect. In the case of the damselfly, *P. inconspicuum*, it was confirmed that *Psi*-AKH and the factor in the CC that had been responsible for the hyperglycaemic effect are one and the same.

The concentration of the injected synthetic peptides in *O. julia* and *P. inconspicuum* could be calculated as the haemolymph volumes (ca. 31 and 7 µl, respectively) had been determined. Injection of 1 pmol of each peptide (enough to elicit a maximum response; Table X; p. 45), corresponds to a haemolymph concentration of 30×10^{-9} M and 140×10^{-9} M, respectively. The peptide concentration in the haemolymph of *P. inconspicuum* is in the same order of magnitude as the $EC_{max.}$ value (130×10^{-9} M) of the endogenous peptide (*Mem*-CC) in *P. sinuata* (Lopata and Gäde, 1994) for a hypertrehalosaemic response. The peptide concentration of *Psi*-AKH is 3.5-fold higher than the $EC_{max.}$ value (40×10^{-9} M) for lipid mobilisation by *Grb*-AKH in the cricket *Acheta domesticus* (Cusinato *et al.*, 1991), 14-fold higher than the $EC_{max.}$ value (10×10^{-9} M) in the tobacco hornworm moth, *M. sexta* (Ziegler, 1990), but ca. 100-fold higher than the response of *L. migratoria* to its own peptide (Cusinato *et al.*, 1991). The significance of the adipokinetic response in the dragon- and damselflies is therefore not clear, and they must join the many AKH/RPCH peptides where there are only hints as to their physiological relevance. However, as injection of low doses of *Ani*-AKH and *Psi*-AKH (smaller quantities than is found of the endogenous peptide in the CC) elicited a significant increase in blood lipids, the peptides appear to be involved in lipid homeostasis. For this reason they were designated *Ani*-AKH, *Anax imperator* adipokinetic hormone, and *Psi*-AKH, *Pseudagrion inconspicuum* adipokinetic hormone.

Although no direct receptor studies on any AKH/RPCH family peptides have been undertaken, it was inferred from bioassays, that in the case of some species and for certain peptides, co-evolution of receptor and ligand had occurred. The charged peptide from Scarabaeid beetles (*Mem*-CC) which has a Tyr^4/Asp^7, is effective in increasing the concentration of trehalose in the haemolymph of *P. sinuata*, when as little as 5 pmol are injected (Lopata and Gäde, 1994). In contrast, almost 500 pmol of *Mem*-CC is required to obtain only 50% of the maximum possible adipokinetic response in *L. migratoria* (Gäde, 1993). The damselfly receptor can recognise similar octapeptides to the native damselfly peptide because all had an adipokinetic effect in *P. inconspicuum* that could not be distinguished from the effect of the native peptide (Table XI; p. 47). A single exchange at position 7 (Gly to Ser; *Lia*-AKH), or changes at positions 5 and 7 (Thr to Ser and Gly to Ser; *Ani*-AKH) and positions 2 and 5 (Val to Leu; Thr to Ser; RPCH) do not prevent binding to the receptor. The adipokinetic effect seen could then be a result merely from the pharmacological action of injecting a relatively high dose, thereby saturating the receptor. The increase in lipid levels in the haemolymph of the damselflies is relatively modest (around 10 mg/ml), and the individual variation is high. Consequently, detecting significant differences between control and experimental groups is very difficult for lower concentrations of peptide used, when the maximum response is not achieved. This is also a likely explanation for the complete failure to establish a dose-response effect in the damselfly bioassay. The alternative explanation for the apparent all or none effect may be that the intracellular cascade of events, leading to mobilisation of lipids into the haemolymph, is only triggered once a certain number of receptor-ligand complexes have been formed. On the other hand, dose-response curves performed in *M. sexta* (Fox and Reynolds, 1991), established that binding of the bioanalogues to the *M. sexta* receptor is apparently not affected by the amino acid substitutions Thr to Ser at positions 5, and Ser to Gly at position 7. The only bioanalogues tested that had substitutions at position 2 (Leu to Val) had many substitutions at other positions as well, markedly decreasing the potency of the bioanalogue in the bioassay (Fox and Reynolds, 1991).

The AKH/RPCH peptides of locusts and cockroaches are synthesised in intrinsic neurosecretory cells of the CC. The incorporation of radioactively labelled Phe into *Lia*-AKH is evidence that the synthesis of *Lia*-AKH occurs in a similar way in *O. julia*.

This confirms the findings of Gäde (1990a) that the adipokinetic peptide (*Lia*-AKH) of the dragonfly *L. auripennis*, is synthesised in its CC.

Some interesting trends emerged when comparing the quantity of *Lia*-AKH stored in the CC of *O. julia* throughout its life cycle. The lowest amounts were measured in the larvae, higher amounts were detected in the teneral (immature) adult and the highest amount was found in the mature adult. These results seem to be contradicted by the magnitude of the increase in the lipids in the haemolymph of locusts after injection of CC extract from larvae of *O. julia* (100% of the maximum possible increase attained), whereas CC extract from adult *O. julia* only attained 38% of the maximum (Table III; p. 30). However, the estimated amount of *Lia*-AKH injected in each instance was quite different: In the case of the larvae 2.9 gland equivalents were injected, amounting to about 24 pmol *Lia*-AKH; only 0.2 gland equivalents of the adults were injected, amounting to about 1.6 pmol *Lia*-AKH. The ED_{50} of Lia-AKH in the locust bioassay is 8.6 pmol (Gäde, 1993), which explains the apparent anomaly.

The content of *Lom*-AKH-I, -II and -III in CC of the locust, *L. migratoria* was shown to increase with age, from the larval stage 2 where the amounts at first were barely detectable, to a maximum value in the oldest locusts (Oudejans *et al.*, 1993). During the aquatic larval stage, the Odonates tend to remain immotile, concealing themselves (Corbet, 1962), and the maximum activity is reached during the mature adult flying phase. If the physiological role of *Lia*-AKH is indeed that of a hormone regulating the supply of fuel for energy demanding activity, then it seems logical that larger quantities need to be stored for putative release during more active stages in the life cycle.

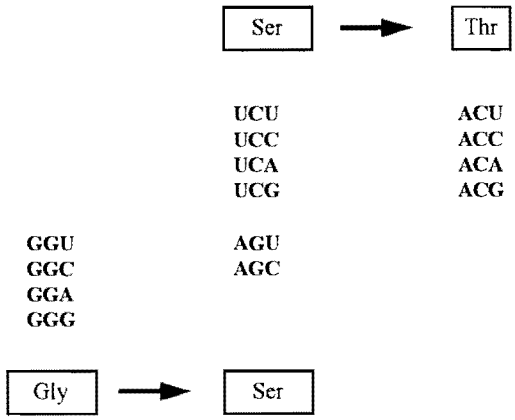
5.2 AKH/RPCH peptides and Odonata phylogeny

Three AKH forms have been shown to occur in the Odonata: *Ani*-AKH and *Lia*-AKH which were sequenced from dragonflies (Anisoptera), and *Psi*-AKH which only occurs in the damselflies (Zygoptera). Although a few insect orders contain two AKH/RPCH family members in their glands, no second peptide with adipokinetic activity was present in any of the Odonata that were screened. This is in agreement

with previous findings in the libellulid *L. auripennis* (Gäde, 1990a). However, the presence of a second AKH peptide cannot be ruled out completely, considering the recent discovery of the more hydrophobic *Lom*-AKH-III in *L. migratoria* (Oudejans *et al.*, 1991). There was also no evidence in any of the dragonflies that were investigated, of the presence of the postulated intermediary peptide, *Xxx*-AKH. All the observed differences in amino acids at position 5 and 7 (from Ser to Thr, and Ser to Gly, respectively) can be explained by point mutations, leading to single base changes when considering the standard genetic code (Fig. 8).

The AKH peptides seem to play an important role in insect physiology and the structural information contained therein has therefore potentially useful application in phylogenetic analyses. Changes in the primary structure of these hormones that disrupt binding to the receptor, would have to be matched by co-evolution of the receptor to which the hormone must bind to deliver its message, if full activity is to be retained. The hyperglycaemic potency of various bioanalogues upon injection into the cockroach, *Blaberus discoidalis*, were compared by investigating the concentration of peptide needed to achieve 50% of the maximal possible response, the so-called ED_{50} (Hayes and Keeley, 1990). Only one amino acid substitution (Leu for Val at position 2), and removal of the C-terminal dipeptide, increased the ED_{50} from 0.6 to 20 pmol compared with the native peptide, *Bld*-HrTH. Binding and recognition of the peptide by the receptor system was therefore disrupted. Full activity could only be restored by injection of higher amounts (more than 100 pmol) of this bioanalogue, compared with a maximal activity that could be achieved using only 3.5 pmol of *Bld*-HrTH (Hayes and Keeley, 1990). The authors suggested that co-evolution had occurred between the hormonal peptide and its cell-surface receptor on the fat body target tissue in *B. discoidalis*, for an optimal "fit" between receptor and its hormone. It can thus be argued that during the evolution of the insects, most base changes leading to amino acid substitutions in AKH peptides would decrease binding to the receptor. These events would have a disadvantageous effect on the insect, by creating an abnormal physiological state, and would be selected against, unless there were a corresponding change in the receptor, to restore binding.

Fig. 8. Amino acid substitutions in AKH peptides of Odonata



The underlying base changes when considering the genetic code are depicted, to explain the observed amino acid substitutions in AKH/RPCH peptides in the Odonata.

The available sequence data for the AKH peptides in the Odonata was therefore evaluated from an evolutionary perspective, in an attempt to draw some conclusions about phylogenetic relationships in the Odonata. A suitable outgroup is necessary, in order to evaluate which character states are plesiomorphic, and which are apomorphic. The ideal candidate in this case is the only non-insect member of the AKH/RPCH peptide family, the crustacean *Pab*-RPCH peptide. The Class Crustacea is classified together with the Class Hexapoda under the subphylum Gnatomorpha, of the Phylum Arthropoda (Boudreaux, 1979). There therefore seems to be considerable justification to select *Pab*-RPCH as outgroup. *Pab*-RPCH differs from the "missing link", the *Xxx*-AKH, only by a substitution of Val by Leu at position 2. Gäde *et al.* (1994) proposed that *Pab*-RPCH is the ancestral peptide of the AKH/RPCH family, based on the most parsimonious trees that could be constructed using the computer programme PROTPARS, taking all known AKH peptide structures into account. The main argument revolves around the identity of the amino acid residue in the ancestral AKH/RPCH molecule, at position 7, where the five amino acid residues (Ser, Gly, Asn, Asp, Trp) observed to occur in AKH/RPCH peptides cannot be explained by single-step point mutations between each other. Two possible routes with either Ser^7 or Gly^7 as the ancestral amino acid at this position were recently proposed (Gäde *et al.*, 1994) with Gly^7 favoured because it is also found in Crustacea (*Pab*-RPCH). The structure of *Psi*-AKH, the novel damselfly peptide, supports this view, because it is

found in members of the family Coenagrionidae, which are generally considered to be ancestral to other Odonata (Fraser, 1957).

There are more taxa than characters that could serve to construct a phylogenetic tree, *de novo,* based only on data from the dragonfly AKH peptide sequences. For this reason, it was interesting to see how well the distribution of the peptide sequences corresponds to current ideas on Odonata evolutionary relationships. Two proposed phylogenies that relate the families of the Anisoptera seem to agree in broad terms (Fig. 9 A & B).

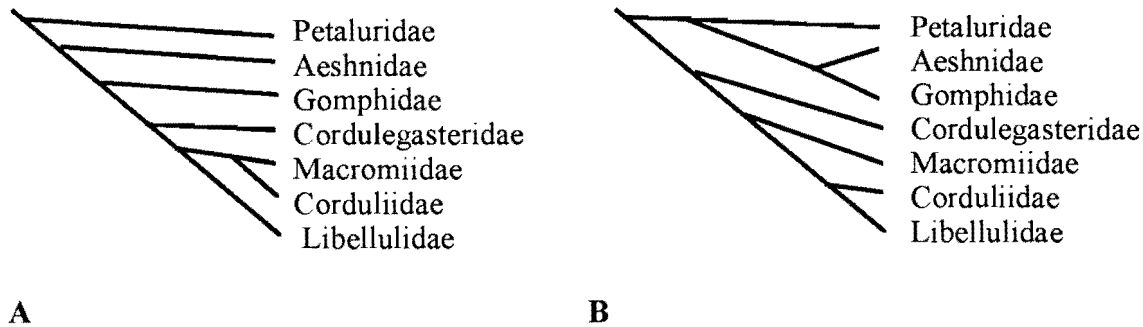


Fig. 9. Phylogenetic relationships in Anisoptera

The hypotheses after Fraser (1957) (A) and Bechly (pers. comm.) (B) are depicted.

More than one point mutation in the Anisoptera must be invoked to explain the occurrence of *Ani*-AKH in the Aeshnidae, Cordulegasteridae, and possibly the Corduliidae and of *Lia*-AKH in the Gomphidae and Libellulidae. Consideration of changes at position 5 (Ser to Thr substitution) indicate that point mutation at position 5 seems to occur relatively easily, which is understandable in view of the highly conservative nature of such a substitution. There is therefore limited value in predicting the phylogenetic relationships of the Anisoptera at the family level.

In order to see whether any prediction could be made when considering suborder level evolutionary relationships, the peptide sequences were fitted to the competing phylogeny of Fraser (1957) and to the controversial phylogeny proposed by Bechly

(1993). In Fig. 10 the variable amino acids are shown for each suborder of the Odonata, with the crustacean *Pab*-RPCH as outgroup and arranged in the two separate phylogenies.

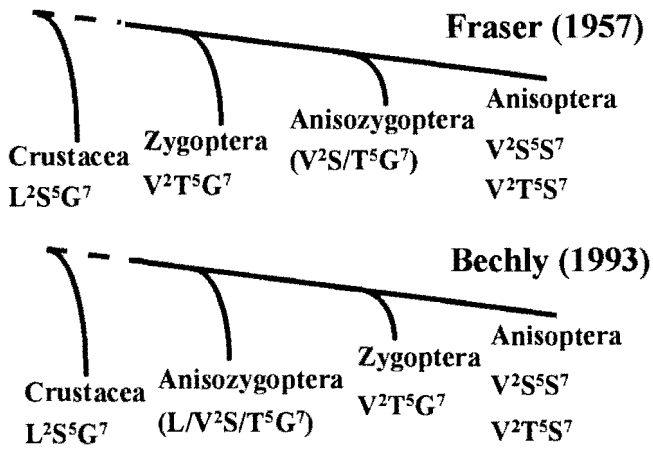


Fig. 10. Odonata phylogenies and AKH/RPCH peptides

Variable positions of the AKH/RPCH peptides in Odonata are superimposed on two proposed phylogenies of Odonata

The possible peptide identity of the suborder Anisozygoptera is unknown (shown in brackets for this reason) as material could not be obtained at the time of writing (Fig. 10). For the purposes of illustration it is assumed that the peptide contains a Gly^7. Two groupings emerge, with one point mutation separating groups into ancestral (or more primitive) and derived (or more advanced), according to whether they contain a Gly, or Ser at position 7. The character state of the outgroup, *Pab*-RPCH is responsible for this polarisation. Which group (Anisozygoptera or Zygoptera) is ancestral in the Odonata, could be decided by the AKH peptide sequence of the Anisozygoptera (which has been unavailable up to now). If this peptide were found to contain a Gly in position 7 (as illustrated), and could therefore be the unknown "intermediary" peptide not found in any insect that has been investigated, the observed peptide sequences fit very well and do not contradict either phylogeny. However, if it were found that the Anisozygopteran peptide contains a Ser at position 7, the traditional phylogeny (Fraser, 1957) represents a more parsimonious solution, and would then be favoured.

If Ser in position 7 has to be fitted to the sequence of events postulated by Bechly (1993), an additional point mutation must be invoked at the Anisozygopteran branch.

A phylogeny cannot be based on one character only. The highly conserved nature of the peptide sequences in odonates as shown by the family-specificity in the Anisoptera and even suborder-specificity in the damselflies (only *Psi*-AKH present in all damselflies investigated, Table XIV; p. 56) shows that successful point mutation in the Odonata has been a rare event, especially taking evolutionary time into account. The fossil record of insects recognisable as members of the Odonata dates back more than 200 million years (Resh and Solem, 1984). This data could therefore be a strong pointer to the dragonfly ancestor.

5.3 Implications of dragonfly morphology

O. julia is a percher and *A. imperator* is a flier, based on the exhibited flight behaviour, as previously discussed. The fresh weights of whole animal, as well as flight muscles, and haemolymph volume of *O. julia, A. imperator, P. inconspicuum and I. senegalensis* are summarised in Table XV, p. 57. The difference in mass of flight muscles in relation to whole body weight measured in *O. julia* (22%) and *A. imperator* (11%) (Table XV) differs from what May (1981) predicted for perchers (45%) and fliers (25%). May (1981) calculated the flight muscle masses indirectly, which may explain the discrepancy. However, the relative values agree. He measured the thoracic volumes of percher and flier dragonflies, and calculated the corresponding flight muscle masses using the muscle density of frogs (as no such data are available for dragonflies). May (1981) speculated that the reduced relative muscle mass of fliers, especially aeshnids, could indicate that maximum short-term power requirements are usually less for species characterised by sustained flight, than for those that typically make repeated sudden take-offs.

5.4 Parameters of energy metabolism

5.4.1 Metabolite concentration in the haemolymph

The open circulatory system of insects leads to relatively large diffusional distances for fuels to reach the target muscle cells. Higher substrate concentrations are therefore necessary to increase the rate of diffusion. In the locust, the initial fuel used is carbohydrate that is present in high concentrations in the haemolymph. To reduce the osmotic effect of such high concentrations, the carbohydrates are present in the form of the disaccharide trehalose and the fatty acids in the form of diacylglycerols. As the lipid concentration (mostly diacylglycerols) in the haemolymph of the locust rises during the first period of flight, it has also been established that the rate of oxidation of diacylglycerol increases (Goldsworthy, 1983; Beenakkers *et al.*, 1984), and therefore the contribution of lipid oxidation to the metabolic rate.

The concentration of a metabolite in the haemolymph may give some indication of its contribution to energy metabolism. Incubation of a working flight muscle preparation from *L. migratoria* with trehalose and lipids resulted in competition between the substrates for the supply of energy, with the degree of carbohydrate utilisation dependent on its concentration (Robinson and Goldsworthy, 1977). The rate at which trehalose or diacylglycerol is oxidised by flight muscles of locusts can be increased, by experimentally elevating the concentration of the particular substrate (Goldsworthy, 1983).

In all the Odonata in this study where the resting concentration of lipids and of carbohydrates in the haemolymph were determined, it was found that the lipid concentration in the haemolymph is higher than that of the carbohydrates. The same is true of the relative concentration of lipids to carbohydrates in the haemolymph of *L. auripennis* (Gäde, 1990a). Thakare *et al.* (1980) determined the chemical composition of larvae of the libellulid *O. chrysis*, with similar results. The concentration of carbohydrates in the haemolymph of this dragonfly amounts to ca. 10 mg/ml, with 95% being contributed by the disaccharide trehalose. The total lipid concentration was found to be 15 mg/ml. It would appear that lipid metabolism is relatively more important for adult dragonflies than for their larvae (the lipid

concentration in adult *O. julia* is 2 to 3-fold higher than in the last instar larvae). The lipid concentration showed a similar trend during development of the dragonfly *O. sabina*, where the lipids increased from around 1.2 mg/ml in the 6th instar (of 13 instars), to 2.2 mg/ml in the adult (Varadaraj, 1979). However, the concentration of total lipids in the haemolymph measured in *O. sabina* seem to be too low by about a factor 10. Larvae of *A. cyanea* have also been shown to take up and incorporate radioactively labelled long chain fatty acids into diacylglycerols in the haemolymph and triacylglycerols in the fat body (Wachtmann and Komnick, 1993). Lipid breakdown is therefore predicted to still play a significant role in the metabolism of the larvae.

Interestingly, there seems to be a correlation between the flight behaviour in the adult dragonflies and the resting concentration of lipids in the haemolymph. The highest concentration of lipids was measured in the flier *A. imperator*, the lowest in the percher damselfly, *P. inconspicuum*, with the percher dragonfly, *O. julia* intermediate (Fig. 6; p. 60). By contrast, the opposite trend seems to occur when considering the concentration of carbohydrates in the haemolymph. As the flight characteristics change from long continuous flight to an increase in the frequency of shorter, trivial flights with long periods of inactivity, the relative contribution of carbohydrates to supply the energy for flight may increase. From observation of the insects flying in the wild, it was obvious that the flight of the dragonfly, *O. julia,* is much more powerful than that of the damselfly, *P. inconspicuum*. Therefore, a more prominent role for carbohydrate metabolism in fuelling flight in the damselfly could be expected. The interpretation of differences in the concentration of metabolites in the haemolymph, in terms of observed differences in energy requirements in the animals, justifies testing other parameters of energy metabolism to support this hypothesis.

The contribution of carbohydrates to the energy requirements, does not appear to change dramatically between the larval and adult stages of the percher, *O. julia*. The ratio of lipids to carbohydrates, is elevated slightly from 2:1 in the larvae to ca. 4:1 in the adult. The contribution of carbohydrates to the energy requirements must certainly decline during adulthood in the flier *A. imperator*, as the ratio of lipid to carbohydrates increases dramatically, from 2:1 in the larvae to 27:1 in the adult. Concomitantly, the absolute concentration of carbohydrates in the haemolymph is decreased as well.

5.4.2 Fuel utilisation during flight

A decrease in the concentration of a substrate in the haemolymph during flight indicates that it may be utilised in the flight muscles. The carbohydrate concentration in the haemolymph of *L. migratoria* decreased during the initial 30 min of flight. The constant concentration of carbohydrates after the initial decrease reflects that a steady-state in the animal has been reached, with utilisation of carbohydrates by the flight muscles balanced by replenishment from substrate stores (Goldsworthy, 1983; Beenakkers *et al.*, 1984). Any calculation of the rate of utilisation of substrates, based only on the changes of the substrate concentration in the haemolymph, is therefore underestimated. One way of circumventing this problem is by the incorporation of a known amount of radioactively labelled substrate and measuring the rate of decline of the specific radioactivity in the haemolymph. The lipid concentration in *L. migratoria* increases after the initiation of flight, reaching a constant level after about 60 min. It was demonstrated, however, that a steady state of utilisation of the diacylglycerols by the flight muscle and release from the fat body, is reached by measuring the decline in the specific radioactivity of labelled [^{14}C]-oleic acid (Beenakkers *et al.*, 1984) according to a method described by Zilversmit (1960). Measuring metabolites usage during flight under experimental conditions may also not reflect "normal" flight behaviour. Dragonflies in the wild have been observed to glide and soar, which means that the power output is greatly reduced, and any reserves of metabolites can be expected to last longer. On the other hand, hovering is thought to be energetically more expensive (Miller, 1987).

The concentration of lipids and carbohydrates in the haemolymph of *O. julia* decreased by 39% and 32%, respectively, after 30 min of flight, indicating that both lipids and carbohydrates are utilised as fuels for flight. The contribution of each of these metabolites to the calculated theoretical O_2 consumption rate is shown in Table XX. The haemolymph lipids are the major contributor (73.8%) to the metabolic rate. The smallest contribution comes from the carbohydrates (12.1%) most of which is also derived from the haemolymph. The small contribution of stored reserves in the flight muscles confirms the generalisation by Bailey (1975) that the quantities of these reserves are sufficient only for the immediate energy requirements at the start of flight,

and not to sustain prolonged flight. After a flight of 2 h the diglyceride content in the flight muscles of *P. flavescens* was significantly reduced, and the non-esterified fatty acid content was significantly increased (Kallapur *et al.*, 1979). Kallapur *et al.* (1979) concluded that the major stored lipids appear to reside in the flight muscle of the dragonfly, as no distinct fat body could be discerned. Unfortunately, they were unable to take haemolymph samples from the dragonflies after flight. The theoretical oxygen consumption of the stored fats in the flight muscle during a flight lasting 120 min was calculated, based on a body mass of 437 mg, the difference of glycerides in a flown versus unflown group as 1.95 mg fat/100 mg flight muscle (Kallapur *et al.*, 1979), and assuming the high value of 25% flight muscle to total body weight proposed for fliers by May (1981). This theoretical rate of oxygen consumption could not have contributed more than 4.5 ml O_2/g body weight/h. to the metabolic rate. Compared with the value reported in Kammer and Heinrich (1978) for *A. grandis* of 17-24 ml O_2/g body weight/h., this implies that the lipid stored in the flight muscle cannot be considered the only major contributor to meet the energy requirement of flying dragonflies.

A high standard deviation of the differences of metabolite concentrations in the haemolymph before and after flight, especially lipids (Fig. 7; p. 63), was observed in *O. julia*. This may indicate that putative release of *Lia*-AKH, and a subsequent adipokinetic effect, could have occurred in some dragonflies, probably depending on their physiological state.

Table XX. Calculated O_2 consumption rates during flight in *O. julia*

		Calculated V_{O2}	% contribution
Haemolymph	Lipids	4.27	73.8
	Carbohydrates	0.64	11.1
	Proline	0.28	4.8
	Subtotal:	5.19	89.7
Flight muscles	Carbohydrates	0.06	1.0
	Proline	0.54	9.3
	Subtotal:	0.60	10.3
Haemolymph + flight muscles	Total:	5.79	100

V_{O2} (expressed as ml O_2/g body weight/h.) for *O. julia* was calculated from metabolites utilised during flight, assuming ATP/g values of 0.5 for lipids, 0.2 for carbohydrates and 0.3 for Pro. P/O_2 ratio of 6 was used. Changes in metabolite content were calculated using an average haemolymph volume (30.5 µl), wet mass of flight muscle (56.2 mg) and whole animal (256 mg), during flight of 30 min.

Proline in the flight muscles of *O. julia* was reduced by 75.4% after 30 min of flight. In the haemolymph the concentration was reduced by 64%, which is similar to the reduction of Pro in the haemolymph (52%) of a gomphid, after flight lasting between 15 and 30 min (Subramanian and Varadaraj, 1985). The initial concentration of Pro they measured (5.6 mM) was approximately half that measured in *O. julia* (ca. 12.4 mM). They reported a very low standard deviation (less than 7% of the mean value) of the concentration of Pro in the haemolymph both before and after flights varying in length between 15 and 30 min, which may indicate that Pro is used early during flight. Interestingly, they found no change in the concentration of Ala as a result of the flight activity. The concentration of Ala that they measured ranged from 3.4 mM in the males, to 1.3 mM in the females. The concentration of Ala measured in *O. julia* was barely detectable (ca. 0.4 mM). The lack of any change in the concentration of Ala in the haemolymph during flight in *O. julia*, and the reduction of the Ala content of the flight muscles after flight, suggests that oxidation of Pro does not occur via the partial oxidation pathway as described by Bursell (1981). Under this model, an increase in the concentration of Ala would be predicted, as Ala is formed during the partial oxidation

of Pro. Such changes in the concentration of Ala and Pro have been observed to occur in insects such as *G. morsitans* (Bursell, 1981), and in *P. sinuata* (Zebe and Gäde, 1993). In *O. julia*, Pro provided a significant contribution to the total calculated metabolic rate (14.1%), predominantly by oxidation of Pro located in the flight muscle (Table XX). The content of Pro in the flight muscle, and in the haemolymph is ca. 5-fold and 6.7-fold less, respectively, than measured in *P. sinuata* (Zebe and Gäde, 1993). The Pro in the flight muscle may therefore represent a store of fuel sufficient to provide energy for trivial flights lasting up to 3 min, based on the contribution of Pro in flight muscles of 10.3% to the total V_{O2}. The role of Pro stores in the flight muscles of *O. julia* may therefore be similar to that of glycogen in flight muscles of *L. migratoria,* where these stores can provide sufficient energy for 2-3 min at the initiation of flight (Goldsworthy, 1983).

The total calculated O_2 consumption rate of 5.8 ml O_2/g body weight/h. in *O. julia* represents a minimum. The rate calculated depends on the time during which the dragonflies were made to fly, in this case 30 min. After ca. 20 min of flight, the dragonflies became exhausted, flying much more weakly. The calculated O_2 consumption rate, based on fuel utilisation during flight could therefore be at least 50% higher at around 8.7 ml O_2/g body weight/h. The lipids in the haemolymph provide the major contribution (73.8 %) to the calculated O_2 consumption rate (Table XX). It has clearly been shown during the adipokinetic bioassay in this dragonfly, that the concentration of lipids in the haemolymph is increased after injection of fractions containing adipokinetic hormone. It is therefore very likely, despite the absence of a clearly defined fat body in adult *O. julia* and *A. imperator* (a fact also noted for *P. flavescens* by Kallapur *et al.* (1979)), that there must be an additional source of lipids, to replenish those oxidised during flight. This is a question that must still be addressed.

The theoretical length of time that an individual of *O. julia* can remain without food, e.g. during spells of cold weather, was calculated using the mass-specific resting metabolic rate of 0.754 ml O_2/g body weight/h. at 30°C measured for *L. auripennis* (May, 1979). The metabolic rate was converted to 10°C assuming a Q_{10} of 2.5. The calculated O_2 consumption rate for *O. julia* is then 30.9 µl O_2/h. When using only that

portion of the metabolites consumed during flight (260 µg lipids, 119.4 µg carbohydrates and 93.5 µg Pro), the total time that the lipids, carbohydrate and Pro reserves will last, was calculated to be 24.5 h with the carbohydrate contribution lasting 2.9 h, the Pro reserves 3.4 h, and the balance contributed by the lipids. May (1979) suggested however, that his "resting" O_2 consumption rates may have been overestimated as it was impossible to eliminate low levels of activity, and therefore this amount of time represents a conservative estimate.

It was of interest to calculate the theoretical length of time that *P. inconspicuum* could stay airborne. Using an average haemolymph volume of 7.1 µl, and assuming an O_2 consumption rate of 17 ml O_2/g body weight/h., the stores of lipids and carbohydrates mobilised as a result of injection of *Psi*-AKH (ca. 69 µg lipids, and 11 µg carbohydrates), the theoretical time available to remain airborne was calculated to be about 11.0 min (the contribution of carbohydrates only calculated as 0.7 min). The relatively small amount of available substrates is clearly the reason for the short, trivial flights that characterise flight behaviour in this insect. It seems likely from these calculations, that the major energy requirements must be met through constant feeding, an idea supported by observations on the feeding behaviour of a related damselfly, *Calopteryx cornelia* (Zygoptera: Calopterygidae). This damselfly makes about 150 feeding flights each day, 43% of which were successful, consuming 6 mg of food, or 11% of its body weight (Higashi, 1973 cited in Corbet, 1980).

Pseudagrion inconspicuum exists on a very restricted energy budget if the preceding examples are taken into account. The theoretical survival time, at resting metabolic rates, when feeding is not possible (e.g. during prolonged cold weather) was therefore calculated. The resting mass-specific metabolic rate for *L. auripennis* was used as before (May, 1979). It was assumed that only that fraction, mobilised as a result of injection of *Psi*-AKH is available during starvation, totalling on average 69 µg lipids and 11 µg carbohydrates per individual. In the case of *P. inconspicuum*, with a body weight of 43.5 mg, it follows that the rate of O_2 consumption at 10°C is 5.25 µl/h. Therefore lipids and carbohydrates would last ca. 24.3 and 1.5 h respectively, totalling a conservative minimum of 25.8 h. No data are available to enable an estimation of the contribution of other metabolites, such as Pro, or glycogen.

The same caveat as discussed during a similar calculation for *O. julia* applies. If this is typical for small damselflies of the family Coenagrionidae, it may help to explain their ability to tolerate all climactic conditions spanning a 3000 m altitudinal gradient (Samways, 1989). Using some of the data available for *A. imperator* (average mass 1032 mg, lipid concentration in the haemolymph 55.3 mg/ml), and assuming that the haemolymph is 12% of fresh weight, the total lipids in one individual is 6.8 mg. If it is assumed that 40% of this is available for energy (i.e. similar to the amount of lipids consumed during flight in *O. julia*), and the resting oxygen consumption of 1.021 ml O_2/g body weight/h. measured for *Anax junius* is used (May, 1979), then in *A. imperator* at 10°C, the rate of oxygen consumption is 168.5 µl/h. This calculation then provides a minimum survival estimate of 30 h without feeding which is higher than in *O. julia*, before other stored metabolites have been taken into account. The family Aeshnidae represented 37% of all species sampled at the highest altitude (3000 m) by Samways (1989). Apart from any other adaptation, the above calculation indicates that an aeshnid such as *A. imperator* is likely to survive longer periods of cold weather when foraging is impossible, than a libellulid such as *O. julia*.

Marden (1989) has presented some interesting data on the benefits and costs associated with the reduction of tissues other than flight muscle, such as the fat reserves. He estimated the time that the dragonfly *Platythemis lydia*, can survive during unfavourable weather when foraging is impossible, to be ca. 2.4 days. This estimate is based, however, on the total depletion of the lipid content of the dragonfly, which seems unlikely.

5.4.3 Mitochondrial oxidation

The mitochondrial membrane is impermeable to NADH (Sacktor, 1975). There is therefore a need to reoxidise the glycolytically formed NADH in the cytosol, and transfer the reducing equivalents to the electron transport chain in the mitochondria. This is achieved by the glycerol-phosphate cycle (Fig. 11) (Zebe *et al.*, 1959). The high capacity for oxidation of α-glycerol-phosphate by isolated mitochondria from *O. julia*, *A. imperator*, as well as *L. migratoria* (Table XVII; p. 59) combined with the almost complete absence of LDH proves once again, that glycolysis in insect flight muscles is

completely aerobic (Sacktor, 1975). The rate of oxidation of α-glycerol-phosphate in *O. julia*, *A. imperator*, and *L. migratoria* is similar to the 16% of the maximal Cytox activity in isolated mitochondria from flight muscles of *S. gregaria* (Suarez and Moyes, 1992). Using a different approach, a similar important role for the α-glycerol-phosphate cycle in *P. flavescens* was proposed after high levels of the enzyme α-glycerol-phosphate dehydrogenase, and very low levels of LDH were measured in its flight muscles (Kallapur and George, 1975). The oxidation of carbohydrates is therefore expected to play a prominent role in the flight metabolism of all of these insects.

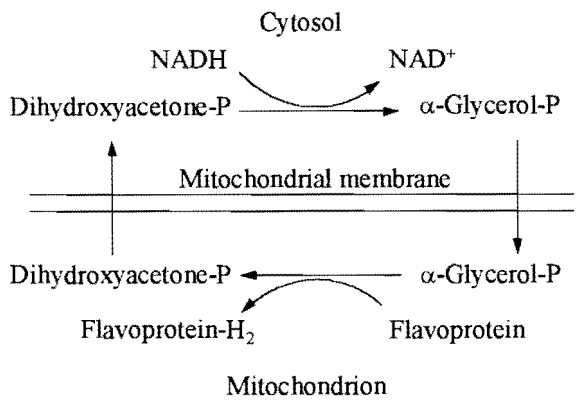


Fig. 11. The α-glycerophosphate cycle

Figure adapted from Sacktor (1975). Transfer of reducing equivalents derived from glycolytically produced NADH in the cytosol into the mitochondrion, is accomplished via the glycerol-phosphate cycle.

The high rates of oxidation of malate by isolated mitochondria from *A. imperator* compared with *L. migratoria* may indicate that in addition to the glycerol-phosphate cycle, there is an active malate-aspartate cycle for the transport of reducing equivalents from the cytosol into the mitochondria. Palmitoyl-carnitine was oxidised by mitochondria of *O. julia*, *A. imperator* and *L. migratoria* at similar rates to pyruvate, indicating a prominent role of lipid oxidation in the flight muscles of these insects. This supports findings by Kallapur and George (1973) which indicated that mitochondrial preparations from flight muscles of the dragonfly *P. flavescens* are capable of oxidising butyrate, octanoate, palmitate, and stearate. They also demonstrated enhanced rates of oxidation of palmitate, on addition of carnitine. Carnitine mediates the transport of

fatty acids to intramitochondrial sites for oxidation. To demonstrate the oxidation in muscles of fatty acid, they are best administered as carnitine-esters (Beenakkers *et al.*, 1967). The 1.5-fold higher mean value of oxidation capacity of palmitoyl-carnitine suggests additional support for the hypothesis that lipid oxidation plays a more prominent role in the provision of energy to the flight muscles of the flier *A. imperator*, than is the case in the percher *O. julia*.

Any precise calculation based on the measured rates of oxidation of substrates by isolated mitochondria must be viewed with caution. A measure of the intactness of the mitochondria is given by the respiratory control ratio (RCR). The RCR is the ratio of the rate of oxidation when stimulated by the presence of ADP (state 3), to the resting rate of respiration when all the ADP has been phosphorylated (state 4) (Estabrook, 1967). The state of coupling of oxidative phosphorylation to electron transport through the respiratory chain can be estimated by measuring the ADP/O ratio, or the disappearance of P_i versus oxygen uptake. The ADP/O ratio and RCR were not determined, as the mitochondrial preparations consisted of many damaged mitochondria (oxidation of NADH was measurable upon addition to the isolated mitochondria). Mitochondria that have been isolated from various sources often require "priming" if maximal oxidation of substrates such as pyruvate is to be sustained. Malate has been used for this purpose (Sacktor, 1975). The primers are generally considered to act by ensuring a supply of Krebs-cycle intermediates, and a requirement for primer would therefore indicate that the isolated mitochondria have suffered a depletion of such substrates during isolation (Slack and Bursell, 1976).

Comparative studies such as establishing whether mitochondria are in fact capable of oxidising particular substrates, or have particular substrate preferences can still be made. In this study it was found that α-glycerophosphate was oxidised at higher rates than pyruvate by isolated mitochondria. Suarez and Moyes (1992) found the opposite to be true. They measured RCR's that were consistently greater than 40 for oxidation of pyruvate + malate as substrates. They do not, however, report RCR values for the oxidation of α-glycerophosphate. The RCR is unlikely to have been as high as with pyruvate + malate, because both intact and partially damaged mitochondria will oxidise α-glycerophosphate at high rates. However, oxidation by damaged mitochondria is

without control, and not responsive to ADP, and if there is any lesion in the Krebs cycle, pyruvate will not be oxidised by such mitochondria, thus having no influence on the magnitude of its RCR. The mitochondrial respiratory rates reported in this study were enhanced by addition of cytochrome C, which indicates that many damaged mitochondria were present in the preparation. Similarly, this may explain why palmitoyl-carnitine was oxidised at rates lower than the rate of 17% of the maximal Cytox activity measured by Suarez and Moyes (1992). The above results must therefore be regarded as preliminary and interpreted with caution.

The possible O_2 consumption rates of *O. julia*, based on the rates of oxidation of various substrates by the isolated mitochondria, can be calculated as they have all been expressed as a percentage of the maximal Cytox activity, and related to the fresh weight. This assumes that the rate of utilisation *in vitro* is the same as the maximum experienced *in vivo*.

The Cytox activity in flight muscles of *O. julia* was measured as 37.6 ± 11.0% O_2/mg/min. Assuming that distilled water contains 5.98 µl O_2/µl, this amounts to 614.5 ml O_2/g/h. The rate of oxidation of pyruvate was measured at 1.82% the maximal Cytox activity, meaning that it alone can account for an O_2 consumption rate of approximately 11.2 ml O_2/g body weight/h. which is similar to that calculated from data of substrate utilisation during flight. Similarly, palmitoyl-carnitine alone, which accounted for 2.34% of the Cytox activity, would represent an O_2 consumption rate of approximately 14.4 ml O_2/g body weight/h. This is in the same order of magnitude as values reported by Kammer and Heinrich (1978) for *A. grandis* (17-24 ml O_2/g body weight/h.). Proline was oxidised at rates similar to pyruvate and palmitoyl-carnitine (2.75%), corresponding to an O_2 consumption rate of approximately 16.9 ml O_2/g body weight/h. The rate of oxidation of each substrate by isolated mitochondria, measured separately, was thus comparable to the rate of oxygen consumption that could be expected during flight. These calculations indicate that the capacity of the mitochondria to oxidise substrates is not a limiting factor in providing sufficient energy to support flight.

5.4.4 Fuels for flight: evidence from the activities of enzymes

Comparative investigations into the maximal activities of enzymes that catalyse reactions that are far displaced from equilibrium in a metabolic pathway, provide information about the possible importance of various catabolic routes. In such investigations, it is assumed that the maximum catalytic activity may be similar to the maximum operation of that pathway in the cell (Crabtree and Newsholme, 1975). Diglyceride lipase activity was reportedly 16-fold higher than the activity of triglyceride lipase in the flight muscles of *A. imperator*, and slightly higher than the maximal activity measured in flight muscles from the locust *L. migratoria* (Crabtree and Newsholme, 1972), indicating a central role for lipid metabolism in this dragonfly. The activity of HOAD is a good indication of the capacity of a muscle to oxidise fatty acids (Beenakkers *et al.*, 1967). The use of this enzyme with the constant proportion approach has been questioned, as its maximal activity is much greater than those of lipase and palmitoyl-carnitine transferase, and is greater than the maximum rates of lipid utilization *in vivo* (Crabtree and Newsholme, 1975).

PFK is an important control site in glycolysis in a number of different organisms, including insects such as locusts (Walker and Bailey, 1969; Wegener *et al.*, 1987). Walker and Bailey (1969) found increases in the activity of PFK with increasing ATP concentration, up to a point where ATP became inhibitory, a phenomenon confirmed in some initial experiments in this study when ATP was added in 10-fold concentration to the assay medium (data not shown). The activity of PFK measured for *L. migratoria* (17.4 ± 1.6 µmol/g/min) corresponds to the value reported by Crabtree and Newsholme (1972) for *L. migratoria* (13.0 µmol/g/min) and *S. gregaria* (17.0 µmol/g/min), but is much lower than values ranging from 22 to 80 µmol/g/min reported for a number of castes in *Bombus* spp. (Newsholme *et al.*, 1972).

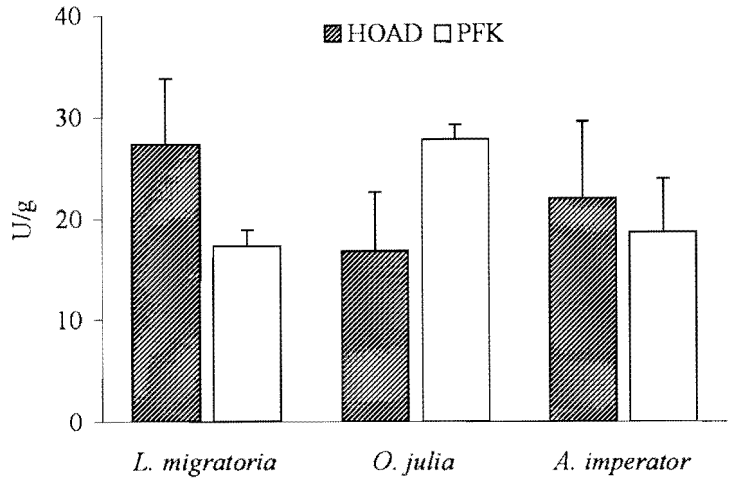


Fig. 12. HOAD and PFK activity in flight muscles

The lower HOAD activity measured in flight muscles of *O. julia*, coupled with a higher activity of PFK relative to that of *L. migratoria* indicates that both carbohydrates and lipids can be oxidised at high rates, with a greater importance of carbohydrate oxidation in *O. julia* (Fig. 12). This is a likely adaptation to the flight behaviour of *O. julia*, which undertakes numerous trivial flights (e.g. to catch prey). Such flights could be fuelled by oxidation of carbohydrates stored in the haemolymph, with presumably fast replenishing of these stores while at rest on its perch. The maximal activities of PFK and HOAD are similar to that found in *L. migratoria,* and it is therefore clear that the flier *A. imperator* contains the biochemical machinery it needs to sustain the rate of oxidation essential for long-term flights (Fig. 12).

The activity measured for CS of *O. julia*, *A. imperator* and *L. migratoria* confirms that flight muscle metabolism in these insects proceeds via the citric acid cycle. The activity of CS was about half that measured in *S. gregaria* (Alp *et al.*, 1976) and *L. migratoria* (Crabtree and Newsholme, 1975), indicating that the extraction method used was probably not optimal.

The activity of glycogen phosphorylase measured in flight muscles of *L. migratoria* (9.3 ± 0.5 µmol/g/min) is similar to the value (7.5 µmol/g/min) reported by Crabtree and Newsholme (1972). In *L. migratoria* this activity is too low to account for the

observed rate of carbohydrate oxidation. Thus, the carbohydrates must be derived from stores other than glycogen in the flight muscles. The very low activities measured in *O. julia* and *A. imperator*, coupled with the low quantities of glycogen stored in the flight muscle of *O. julia*, demonstrate a very low dependence on glycogen (stored in the flight muscles) as energy substrate during flight in dragonflies. Insect flight muscle in general has lower activity of glycogen phosphorylase than found in white muscle of vertebrates, where a larger dependence exists on provision of ATP from anaerobic glycolysis, during breakdown of glycogen (Crabtree and Newsholme, 1972).

The activity of LDH measured in flight muscles (1.1 ± 0.5 µmol/g/min) is similar to that reported (1.6 µmol/g/min) in Crabtree and Newsholme (1972). The almost complete absence of LDH activity from flight muscles of *L. migratoria, O. julia* and *A. imperator* indicates that anaerobic metabolism does not make any contribution in supporting the energetic requirements in these insects.

The maximal activities of GAPDH and PK are much greater than the activity of PFK in *L. migratoria, O. julia* and *A. imperator.* The activity of GAPDH has been used as an index for glycolysis in the constant-proportion approach, but its activity is much greater than the flux through glycolysis *in vivo* (Crabtree and Newsholme, 1975). Despite the fact that the reaction catalysed by PK is irreversible *in vivo*, it is not suitable as an indicator for the maximum rate of glycolysis (Crabtree and Newsholme, 1975). The presence of high activities of these enzymes, however, confirms that glycolysis can play an important role in energy supply of the dragonflies studied.

5.5 Implications for future research

Efforts are being made to obtain CC material from a representative of the Anisozygoptera, *Epiophlebia superstes*. Identification of the AKH peptide of this species may shed some light on the phylogenetic relationship of this group with respect to the Zygoptera and the Anisoptera.

Further studies into the partitioning of metabolites in *O. julia* will be undertaken, in order to augment the available data, to calculate a complete and accurate energy budget and to enable comparison with other Odonata. Where do the lipids that are mobilised by the adipokinetic hormone come from?

A large problem remains in trying to assign hormonal status to the AKH/RPCH peptides. In most cases release of the peptides during flight has not been demonstrated, which places a question mark over statements concerning a postulated physiological role for these peptides. The first evidence for the actual release of the hormone in locusts was obtained after higher adipokinetic activity was isolated from the haemolymph of flown, than that from unflown locusts (Cheeseman *et al.*, 1976). The availability of radioactively labelled hormone would enable the titre of the hormone in the haemolymph to be more accurately determined, providing an improvement in demonstrating peptide release. Additionally, experiments involving cardiectomised dragonflies may shed some light on the influence that the CC have on the concentration of metabolites in the haemolymph *in vivo* and whether this affects the ability of the dragonflies to sustain flight in any way. The feasibility of such experiments is being investigated.

A better method for isolation of coupled, intact mitochondria from the flight muscles of the dragonflies is in preparation. Possible differences between perchers and fliers in the capacity of their mitochondria to oxidise substrates such as palmitoyl-carnitine will be further investigated.

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