FUELS FOR FLIGHT IN THE FRUIT BEETLE, *PACHNODA SINUATA*, AND CONTROL OF FLIGHT METABOLISM

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ABSTRACT

Isolated flight muscle mitochondria of *Pachnoda sinuata, Decapotoma lunata, Trichostetha fascicularis, Lepithrix* sp. and *Camenta innocua* prefer the oxidation of proline, pyruvate and α-glycerophosphate, while those of *Locusta migratoria* prefer the oxidation of palmitoyl-carnitine, pyruvate and α-glycerophosphate. Palmitoyl-carnitine cannot be oxidised directly by *P. sinuata* flight muscle mitochondria, while proline is oxidised at low rates in locust mitochondria. At low concentrations of proline, the respiration rate during co-oxidation of proline and pyruvate is additive, while at high proline concentrations it is equal to the respiration rates of sole proline oxidation.

Flight muscles of *P. sinuata* and *D. lunata* were found to have high activities of the enzymes alanine aminotransferase and NAD-dependent malic enzyme which are involved in proline metabolism, while the activities of these enzymes were lower in locust flight muscles. The activity of 3-hydroxyacyl-CoA dehydrogenase, an enzyme used in fatty acid oxidation, is low in the flight muscles of *P. sinuata* and *D. lunata*, but high in locust flight muscles. Enzymes involved in carbohydrate breakdown (glyceraldehyde-3-phosphate dehydrogenase, glycogen phosphorylase) were found to have high activities in flight muscles of *P. sinuata, D. lunata* and *L. migratoria*.

Two methods of tethered flight were investigated. One of these allowed the animals to produce lift. During lift generating flight, proline concentrations in haemolymph and flight muscles of *P. sinuata* decrease sharply with concomitant increases in alanine concentrations. During recovery after flight, proline concentrations increase while concentrations of alanine decrease. Haemolymph carbohydrate concentrations increase during the first seconds of flight but decline consistently thereafter. During a subsequent rest period concentrations again increase. Glycogen concentration in the flight muscles decrease sharply in the first few seconds of flight, gradually declining thereafter. During subsequent recovery, flight muscle glycogen concentrations increase. Lipid haemolymph concentration increase only slightly during flight and rest thereafter.

Two distinct metabolic phases were observed during lift generating flight. The first phase is characterised by a rapid change of proline and alanine levels in the haemolymph and flight muscles and changes in the glycogen of the flight muscles. Carbohydrates are released from the fat body into the haemolymph and oxygen consumption is high. During the second phase of flight, carbohydrates previously released into the haemolymph are oxidised while changes

of proline and alanine in haemolymph and flight muscles and glycogen in the flight muscles are minor. Oxygen consumption is lower.

During flight without lift generation metabolic changes are several times slower compared with those of lift producing flight. Beetles are able to fly about seven times longer under these conditions than during flight with lift generation.

Resting basalar (BM), dorso-ventral (DVM) and dorso-longitudinal (DLM) flight muscles showed no differences in levels of proline, alanine and glycogen. After different periods of flight on a flight mill, when lift and wing loading are minimised, DVM was found to have the highest levels of proline and glycogen, but the lowest levels of alanine when compared with BM and DLM. There was no evidence to suggest that different flight muscles are specialised for either proline or carbohydrate utilisation.

Oxygen consumption calculated from data of lift generating flight showed that proline and carbohydrates make equal contributions to the energy supply for the flight muscles. Flight muscle stores contribute 54% and those of the haemolymph 46 % to the overall energy consumed during flight.

Haemolymph proline concentrations of *P. sinuata* were found to follow a circadian pattern, with maximum levels recorded in the morning hours and minimum levels recorded at around midnight.

P. sinuata was found to elevate its thoracic temperature (T_{th}) prior to take-off either by basking in the sun or endothermically to within a narrow range, around 33 °C. During flight at low ambient temperatures (T_a), T_{th} drops steadily till it reaches 28 °C, which is insufficient to sustain flight, while T_{th} remains stable during flight at high T_a. Wing beat frequency, however, is dependent on T_{th}, with wing beat frequency decreasing, in a linear relationship, with decreasing T_{th}. During endothermic warm-up the oxygen consumption is 45 % of that rate achieved during lift generating flight. It is, however, similar to the oxygen consumption rate during flight without lift production.

The energy for endothermic warm-up was found to be produced solely through the oxidation of proline. Carbohydrate concentration in flight muscles and haemolymph is not affected by the warm-up. In contrast, proline concentrations decrease and alanine concentrations increase in the flight muscles and haemolymph during warm-up, while haemolymph lipids do not change.

Measurements of the respiratory quotient (RQ: 0.9) confirmed that *P. sinuata* uses a combination of carbohydrates and proline during rest and flight with and without lift

production. During warm-up the RQ is significantly lower at 0.82, closer to the theoretical value of 0.8 for the partial oxidation of proline.

Flight experiments with *D. lunata* and *T. fascicularis* demonstrate that these beetles use a combination of both proline and carbohydrates as fuels for flight. Determination of haemolymph metabolite changes and RQ during flight showed that the Carpenter bee, *Xylocopa capitata*, uses only carbohydrates to power flight.

Starvation of *P. sinuata* of up to 30 days causes a rapid decrease of carbohydrates and alanine in the flight muscles, fat body and haemolymph, while the respective proline concentrations remain high and haemolymph lipid concentrations do not change. Beetles are able to fly throughout the starvation period. During this flight only changes in proline concentrations occur.

The endogenous AKH peptide of *P. sinuata*, Mem-CC, was found to cause a hyperprolinaemic effect which is both dose- and time-dependent. The maximum response of proline concentrations to the action of this peptide is limited by feedback inhibition of proline.

Sructure activity studies suggest that position seven of the peptide is important for receptor recognition. Furthermore, the hyperprolinaemic effect seems to be mediated by a different receptor than the hypertrehalosaemic effect.

Two novel AKH peptides were found in *P. sinuata* and *T. fascicularis*, the sequences being: pGlu-Ile-Asn-Leu-Thr-XAA-Gly-Trp (*P. sinuata*) and pGlu-Ile-Asn-Leu-Thr-XAA-Gly-Trp (*T. fascicularis*). The amino acid in position 6 (XAA) is, in both peptides, as yet unclear.

During the oxidation of proline, isolated flight muscle mitochondria of *P. sinuata* produced equimolar amounts of alanine. Concomitant rates of oxygen consumption during this process lead to the conclusion that proline is oxidised partially according to:

$$\text{proline} + 2.5\ O_2 + 14\ \text{ADP} + 14\ P_i \rightarrow \text{alanine} + H_2O + 2\ CO_2 + 14\ \text{ATP}.$$

Proline is re-synthesised, *in vitro*, from alanine and acetyl-CoA in the fat body of *P. sinuata*. High activities of the enzymes thought to be necessary for proline re-synthesis were present in this tissue. The production of proline from alanine by *P. sinuata* fat body *in vitro* is equimolar suggesting that re-synthesis of proline follows the equation:

$$\text{alanine} + \text{acetyl-CoA} + \text{NADH} + H^+ \rightarrow \text{proline} + H_2O + NAD^+ + \text{CoAH}.$$

The source of the acetyl-CoA for this synthesis of proline is the lipid stores of the fat body of *P. sinuata*.

ABBREVIATIONS

Amino acids:

Ala	alanine
Arg	arginine
Asn	aparagine
Asp	aspartate
Gly	glycine
Ile	isoleucine
Leu	leucine
Met	methionine
Phe	phenylalanine
Pro	proline
Ser	serine
Thr	threonine
Trp	tryptophan
Tyr	tyrosine
Val	valine

Other abbreviations:

ADP	adenosine-5'-diphosphate
AKH	adipokinetic hormone
AlaT	alanine aminotransferase
AMP	adenosine-5'-monophosphate
Ani–AKH	*Anax imperator* adipokinetic hormone
AspT	aspartate aminotransferase
ATP	adenosine-5'-diphosphate
BM	basalar muscle
BSA	bovine serum albumin
Cam-HrTH-I	*Carausius morosus* hypertrehalosaemic hormone I
Cam-HrTH-II	*Carausius morosus* hypertrehalosaemic hormone II
cAMP	cyclic AMP

CC	corpus cardiacum, corpora cardiaca
CoA	coenzyme A
cpm	counts per minute
Del-CC	*Decapotoma lunata* corpus cardiacum peptide
dH_2O	distilled water
DLM	dorso-longitudinal muscle
DTT	dithiothreitol
DVM	dorso-ventral muscle
EDTA	ethylenediaminetetraacetic acid
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GluDH	glutamate dehydrogenase
gfw	gram fresh weight
HOAD	3-hydroxyacyl-CoA dehydrogenase
Lom-AKH-I	*Locusta migratoria* adipokinetic hormone I
Lom-AKH–III	*Locusta migratoria* adipokinetic hormone III
MALDI	matrix-assisted laser desorption/ionisation
MDH	malate dehydrogenase
ME	malic enzyme
Mem-CC	*Melolontha melolontha* corpus cardiacum peptide
NAD^+	nicotine adenine dinucleotide
NADH	nicotine adenine dinucleotide (reduced)
NADP	nicotine adenine dinucleotide phosphate
NADPH	nicotine adenine dinucleotide phosphate (reduced)
ODM	oblique dorsal muscle
Ona-CC	*Onitis aygulus* corpus cardiacum peptide
Pab-RPCH	Pandalus borealis red pigment concentrating hormone
Pas-CC-II	*Pachnoda sinuata* corpus cardiacum peptide II
PCA	perchloric acid
Pea-CAH-I	*Periplaneta americana* cardioacceleratory hormone I
Pea-CAH-II	*Periplaneta americana* cardioacceleratory hormone II
pGlu	pyroglutamate, 5-oxopyrrolidine-2-carboxylic acid
Pht–HrTH	*Phormia terraenovae* hypertrehalosaemic hormone
ProDH	proline dehydrogenase

PTH	phenylthiohydantoin
RCR	respiratory control ratio
RPCH	red pigment-concentrating hormone
RP-HPLC	reversed phase high performance liquid chromatography
RQ	respiratory quotient
Scd-CC-I	*Scarabaeus deludens* corpus cardiacum peptide I
Scd-CC-I	*Scarabaeus deludens* corpus cardiacum peptide II
SM	subalar muscle
STP	standard temperature and pressure
StPM	sternopleural muscle
T_a	ambient temperature
TCxM 1	tergocoxal muscle 1
Tem-HrTH	*Tenebrio molitor* hypertrehalosaemic hormone
TFA	trifluoroacetic acid
TLC	thin layer chromatography
TOF	time of flight (mass spectrometry)
TRA	triethanoleamine
Trf-CC-II	*Trichostetha fascicularis* corpus cardiacum peptide II
T_{th}	thoracic temperature
U	enzyme units (µmol product formed per min)
UL	universally labelled
V_{O_2}	oxygen consumption
WBF	wing beat frequency

1. INTRODUCTION

Many insects have developed the ability to fly over various distances and for different durations. In some insects, flight plays an important role in their life cycle. Locusts, for example, perform long-distance migratory flights to locate food sources and breeding sites. During these migrations they form large swarms which cause enormous damage to crops (Chapman, 1976; p. 23-24). Other insects migrate on a smaller scale. One example is the African fruit beetle, *Pachnoda sinuata*. Its larvae live underground and after the adult moult, the beetles have to fly to their food plants. Although these beetles do not migrate in the true sense, their ability to fly has aided their distribution. Originally abundant in the Transvaal, *P. sinuata* is now also very common in gardens and fruit plants of the Western Cape Province of South Africa (Holm and Marais, 1992).

1.1. Energy substrates for insect flight

Regardless of its duration, lift generating flight is very energy demanding. Due to the fact that insect flight muscles perform completely aerobically, oxygen consumption can be used as a good measure to estimate the energy expense of insect flight. When flight commences, oxygen consumption can increase more than 100-fold and values ranging from 40 to 150 ml O_2/g x h have been measured during free hovering flight in various insects (Casey, 1989). As a result, insect flight muscles are one of the metabolically most active tissues in nature. The high energy demands during flight are met through oxidation of lipids, carbohydrates and the amino acid proline. While carbohydrates can be used in combination with lipids or with proline, no case has yet been reported for a oxidation of lipids in combination with proline.

Lipids are the major fuel for long-term flights in insects such as *Locusta migratoria* (Jutsum and Goldsworthy, 1976) and the moth *Manduca sexta* (Ziegler and Schulz, 1986a). They are also the energy substrate in insects which do not feed as adults, such as the moth *Philosamia cynthia* (Beenakkers, 1969). In both long-term flight and non-feeding, the storage and use of lipids is advantageous, because lipids are the least oxidised of all possible energy substrates and, in contrast to glycogen, they can be stored without being hydrated.

Lipids are mainly stored in the fat body as triglycerides (Wheeler, 1989). In a multi-step procedure, these triglycerides are converted into diglycerides during flight and released into the haemolymph where they are loaded onto a lipoprotein carrier (for details see Beenakkers *et al.*, 1984). When arriving at the flight muscles, the diglycerides are released from the carrier and hydrolysed. The resulting fatty acids can be oxidised after a carrier (carnitine) has transported them across the mitochondrial membrane (see Crabtree and Newsholme, 1975).

The use of carbohydrates as a fuel for flight is widespread amongst the insects. Many hymenopterans and dipterans are well known for their exclusive use of carbohydrates to power flight (Sacktor, 1975). In locusts (Jutsum and Goldsworthy, 1976) and certain moths, for example *Manduca sexta* (Ziegler and Schulz, 1986a,b), carbohydrates are oxidised during short-term flight and in the initial stages of long-term flight. Later, however, these insects switch to the use of lipids, and the energy yield from carbohydrate oxidation becomes minor.

Carbohydrates are stored as glycogen in the flight muscles and the fat body. The breakdown of fat body glycogen during flight is catalysed by glycogen phosphorylase, which shortens the glycogen chains by single glucose units, resulting in the formation of glucose-1-phosphate. The latter is converted via several steps to trehalose (Candy and Kilby, 1961) and released into the haemolymph. Trehalose is subsequently converted into two glucose units by the action of the enzyme trehalase (Sacktor, 1975). Thereafter, the glucose enters the glycolysis and its carbon units are oxidised in the Krebs cycle.

In many insects proline is present in haemolymph and flight muscles (Wheeler, 1989). As early as 1963 it was shown by Bursell that proline serves as an energy substrate during flight in the tsetse fly, *Glossina morsitans*. Since then, several investigations have led to the conclusion that proline is partially oxidised to supply the flight muscles with energy and that alanine is the end product of this partial oxidation (see Bursell, 1981). However, in some insects there are indications that a portion of proline is completely oxidised: in the Japanese beetle, *Popillia japonica* (Hansford and Johnson, 1975) and some dragonfly species (Subramanian and Varadaraj, 1985; Janssens, 1995) the consumption of proline does not result in an equimolar appearance of alanine.

The degree of proline utilisation for flight ranges from the 'sparker' function proposed for the blowfly, *Phormia regina* (Sacktor and Wormser-Shavit, 1966; Sacktor and Childress, 1967), via the combined use of proline and carbohydrates in the Colorado potato beetle, *Leptinotarsa decemlineata* (Weeda *et al.*, 1979), and the African fruit beetle, *Pachnoda sinuata* (Zebe and Gäde, 1993), to the exclusive breakdown of proline in the tsetse fly (see Bursell, 1981) and possibly some scarab beetles (Gäde, 1997a,b).

Although high concentrations of proline can be found in haemolymph, flight muscles and fat body of insects, there are no known specialised storage organs for proline and it must therefore be provided through synthesis during flight. The fat body was found to be the site where this re-synthesis of proline takes place in the tsetse fly (McCabe and Bursell, 1975, Bursell, 1977) and the Colorado potato beetle (Weeda *et al.*, 1980b). The alanine generated during breakdown of proline, is in turn the precursor for its re-synthesis (see section 1.4.)

Various direct and indirect experimental methods can be applied to establish which fuels are oxidised during flight in a particular insect. A simple way to obtain some information is to measure the resting levels of potential substrates in the haemolymph and flight muscles. High concentrations of a flight substrate in these organs can provide evidence that this fuel is likely to be used to power flight. For example, in locusts, the concentration of carbohydrates in the haemolymph is very high (around 32 mg/ml) and has been shown to be used in the first minutes of flight (Jutsum and Goldsworthy, 1976). Another example are dung beetles of the genus *Onitis*, which have negligible amounts of carbohydrates in their haemolymph and flight muscles, but contain high concentrations of proline. It is therefore believed that these beetles break down proline exclusively during flight (Gäde, 1997a).

However, this method cannot generally be used to detect potential lipid users. Although some of these lipid users, such as the moth, *Hippotion eson*, have very high resting values of haemolymph lipids (Liebrich and Gäde, 1995), in others, such as the locust, resting lipid levels are relatively low, increasing only during flight and in particular, during rest thereafter (Jutsum and Goldsworthy, 1976).

These first indications from resting fuel levels must be substantiated by other methods such as the measurement of metabolite changes in haemolymph, flight muscles and fat body and the determination of the respiratory quotient (RQ) during flight. The

gas exchange during insect flight is almost entirely due to the action of the flight muscles, while the respiration of other tissues becomes negligible. Therefore, the RQ of an insect can be used to collect some information about possible substrates oxidised by its flight muscles. Furthermore, no prior knowledge of the biochemical pathways involved is necessary.

Apart from these direct measurements *in vivo*, several indirect methods can be employed. One such a method is the comparison of maximum activities of enzymes involved in particular metabolic pathways, which provide energy during flight. Maximum activities of so-called non-equilibrium enzymes, enzymes which catalyse the rate-limiting step of a pathway, can be helpful to determine the metabolic flux of certain pathways and their significance can be compared (Crabtree and Newsholme, 1975; Newsholme and Crabtree, 1986). Other authors have used the activities of so-called constant-proportion enzymes to reach conclusions as to which fuel is being used (according to Pette, reviewed in Beenakkers *et al.*, 1984). These enzymes are near-equilibrium enzymes of pathways, describing the potency of a particular metabolic route and are of great value for the metabolic evaluation of insect flight muscles.

Determination of respiration rates for mitochondria isolated from flight muscles with different potential substrates is yet another indirect method to examine the same problem. The substrate which is preferably oxidised *in vitro* is most likely to also be used *in vivo* to power flight. Such experiments can also give some information about the pathways involved in the breakdown of the particular fuel. For example, this method was used to provide evidence that proline is an important fuel for flight in the Colorado potato beetle (de Kort *et al.*, 1973).

Finally, radioactively labelled substrates can be applied and the incorporation of the label into metabolic intermediates and end products can be measured. Using such experiments it is possible to confirm indications about potential flight substrates, which resulted from the above mentioned methods. Moreover, the biochemical route of their breakdown can be traced.

For a convincing statement about an insect's energy substrates for flight, it is necessary to apply a variety of the above mentioned methodologies. The results are especially meaningful when they can be compared with those of insects with known metabolic fuels and whose flight physiology is well studied.

1.2. The importance of flight muscle temperature for flight

Insects are highly dependent on the environmental temperature. They experience extreme temperatures and temperature fluctuations in space and time throughout their life cycle. Endothermic regulation of flight muscle temperature is therefore a great advantage to reduce dependency on ambient temperature (T_a). This ability to actively regulate body temperature is known from various kinds of insects (see Heinrich, 1993), although the extent of endothermy often ranges greatly within an insect group (see Heinrich, 1993). For example, some moths such as *Operophtera bruceata*, are not endothermic and are able to fly with muscle temperatures of close to 0 °C, while others, such as *Manduca sexta*, regulate their thoracic temperature (T_{th}) at about 40 °C during flight and can generate the maximum lift only when this temperature is reached and maintained (Heinrich, 1993).

Not surprisingly, a wide range of thermoregulatory ability is also found in the largest of the insect groups, the Coleoptera. While some flying beetles, such as tiger beetles, show no endothermic warm-up and can fly only when T_a is sufficiently high (Morgan, 1985), others, such as many members of the superfamily Scarabaeoidea, have the ability to elevate their body temperature endothermically (Heinrich, 1993). In some species of the subfamily Scarabaeinae (true dung beetles) the speed of dung ball making depends on the T_{th} of the individual. For example, Bartholomew and Heinrich (1978) found that the velocity of ball making by *Scarabaeus laevistriatus* is four-times higher at a T_{th} of 43 °C compared with a T_{th} of 28 °C. The authors showed that this elevated T_{th} is a result of endothermy rather than a by-product of activity.

Flower scarabs (subfamily Cetoniinae) are fast-flying beetles which feed mainly on all parts of flowers and on ripe fruit (Donaldson, 1985). Several members of this group have been shown to warm up prior to flight (Nicolson and Louw, 1980; Chappell, 1984; Heinrich and McClain, 1986). However, one species, *Pachnoda sinuata*, regulates its T_{th} by means of basking in the sun rather than by heating up endothermically, of which it is capable. This behavioural thermoregulation probably lowers the energetic cost. Thus, the beetles remain at a low T_{th} when foraging despite their potential to elevate their body temperature actively. It was in this context, that Heinrich and McClain (1986) called *P. sinuata* endothermically 'lazy'. During flight, *P. sinuata* is unable to maintain the elevated T_{th} when T_a is too low and consequently stops flying (Heinrich and McClain, 1986). Although there is some indication that one cetoniine scarab, the green fig beetle,

Cotinus texana, can regulate its T_{th} during flight (Chappell, 1984), this has not yet been confirmed for other members of the group.

The highest level of endothermy amongst the Coleoptera is found in rain beetles of the genus *Plecoma*. Males fly even at T_a of -1 °C in winter nights, during which they have a T_{th} of close to 40 °C (Morgan, 1987). Endothermic warm-up before flight is achieved by contraction of the flight muscles (Heinrich, 1993). In the moth, *Manduca sexta*, carbohydrates from haemolymph and flight muscles provide the energy during this period (Joos, 1987), however, in most cases, it is not known what fuels are involved and what organs provide them. Some authors have suggested the involvement of a 'futile' substrate cycle in thermogenesis. The presence of such cycles was indeed confirmed in *Bombus* spp. (Newsholme *et al.*, 1972) and *Acherontia atrops* (Surholt and Newsholme, 1983). To what extent these cycles contribute to the overall energy budget during warm-up, however, has yet to be demonstrated.

1.3. AKH peptides

In insects, neuropeptides play an important role in cell communication (see Gäde, 1997c). One group of these peptides regulates metabolic functions and homeostasis. The Adipokinetic Hormone/Red Pigment-Concentrating Hormone family (AKH/RPCH family) is part of them. While the only known RPCH so far is Pab-RPCH (nomenclature after Raina and Gäde, 1988), a chromatophorotropin from eyestalk glands of crustaceans (Fernlund and Josefsson, 1972), more than 30 structurally different AKHs have been fully characterised from insect corpora cardiaca (Gäde, 1990a, 1996). Members of the AKH/RPCH family have the following features in common: they are octa-, nona- or decapetides and are blocked at the N-terminus as well as at the C-terminus. The N-terminus is protected by a pyroglutamate residue, while the C-terminus is blocked by amidation of the free carboxylic group. This protects the peptides from digestion by exopeptidases. All members of the family have aromatic amino acids at least in position 4 and 8, and all but three are not charged. In Table 1 the different amino acids found for each position are presented. Trp is always found in position 8, while Gly occupies position 9. This is also the case in octapeptides, because the prohormone contains Gly at this position, which is used for amidation (O'Shea and Rayne, 1992).

Table 1. 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	
	Tyr				Ala	Asp				
	Phe					Trp				
						Val				

Gäde (1987, 1989) demonstrated that a HPLC fraction of corpora cardiaca from *Pachnoda* and *Melolontha* species contained bioactivity when tested in cockroaches and locusts. Moreover, the peptidic nature of the material was shown and its amino acid composition determined (Gäde, 1989). The same material was subsequently purified from *Geotrupes stercorosus* and *Melolontha melolontha* and the following sequence was obtained (Gäde, 1991):

pGlu - Leu - Asn - Tyr - Ser - Pro - Asp - Trp - NH_2

The novel peptide was code-named ***Melolontha melolontha*-CC (Mem-CC)**. Mem-CC is one of the few members of the AKH/RPCH family which is charged (Asp^7). Up until now it has been found in a member of melolonthine (*M. melolontha*; Gäde, 1991), some cetoniine scarabs (*P. sinuata, Trichostetha fascicularis, T. albopicta;* Gäde *et al.*, 1992a) and in one geotrupid, *Geotrupes stercorosus* (Gäde, 1991).

While no effect of this peptide was found in conspecific bioassays with *M. melolontha* (Gäde, 1991), it caused a small hypertrehalosaemic effect in *P. sinuata* (Lopata and Gäde, 1994). Injected into acceptor locusts and cockroaches, the peptide slightly elevated the concentration of haemolymph lipids and carbohydrates, respectively (Gäde, 1991). Injection of crude extracts of corpora cardiaca of *P. sinuata* resulted in hyperprolinaemia in a conspecific bioassay, but this activity could not be linked to Mem-CC (Lopata and Gäde, 1994). In fact, AKH peptides were only recently confirmed in scarabaeine and onitiine dung beetles as being the active principle behind the elevation of haemolymph proline concentration (Gäde, 1997a,b). One of these AKH peptides, Scd-CC-I, is only different in position 2 when compared with Mem-CC (see Table 2). Besides Mem-CC, several other AKH peptides have been isolated and fully characterised from coleopterans (Table 2).

Table 2. Primary structures of AKH peptides found in beetle species.

Mem-CC	pGlu – Leu – Asn – Tyr – Ser – Pro – Asp – Trp – NH_2
Ona-CC-I	pGlu – Tyr – Asn – Phe – Ser – Thr – Gly – Trp – NH_2
Scd-CC-I	pGlu – Phe – Asn – Tyr – Ser – Pro – Asp – Trp – NH_2
Scd-CC-II	pGlu – Phe – Asn – Tyr – Ser – Pro – Val – Trp – NH_2
Pea-CAH-I	pGlu – Val – Asn – Phe – Ser – Pro – Asn – Trp – NH_2
Pea-CAH-II	pGlu – Leu – Thr – Phe – Thr – Pro – Asn – Trp – NH_2
Del-CC	pGlu – Leu – Asn – Phe – Ser – Pro – Asn – Trp – Gly – Asn – NH_2
Tem-HrTH	pGlu – Leu – Asn – Phe – Ser – Pro – Asn – Trp – NH_2

For explanation of peptide names see Abbreviations.

Binding of an AKH peptide to its receptor is a pre-requisite for hormonal action. Structure-activity experiments injecting closely related peptides and/or their synthetic analogues, which have, as yet, not been found in nature, into the same acceptor species can give some information about the structural characteristics of a peptide which are required for receptor recognition (Gäde, 1992a, 1993).

1.4. Metabolic pathways of proline during insect flight

Results of several investigations suggest that proline serves as flight substrate in a number of insects. However, biochemical pathways of proline oxidation and its re-synthesis have only been fully described for the tsetse fly (see Bursell, 1981) and partially described for the Colorado potato beetle (Weeda *et al.*, 1980a,b). The whole process is depicted in Figure 1. In brief, during flight proline is degraded in the flight muscles in two steps to glutamate, the first step being catalysed by proline dehydrogenase. Glutamate subsequently serves as the substrate for alanine aminotransferase. This enzyme makes the carbon skeleton of glutamate available for oxidation in the Krebs cycle in the form of α-ketoglutarate. The 5-carbon moiety is only partially oxidised. Malic enzyme decarboxylates malate from the cycle and supplies a 3-carbon unit in the form of pyruvate. Pyruvate subsequently acts as the acceptor for the ammonia released from glutamate by the action of alanine aminotransferase, resulting in the formation of alanine. The latter is released into the haemolymph and travels to the fat body where it serves as the precursor for the re-synthesis of proline. The additional two carbon units necessary for this re-synthesis are supplied by the lipid stores in the fat body of the tsetse fly

(McCabe and Bursell, 1975; Bursell, 1977) and most likely in the Colorado potato beetle (Weeda *et al.*, 1980b). The two overall reactions which describe the oxidation (1.) and re-synthesis (2.) of proline are:

1. Pro + 2.5 O_2 + 14 ADP + 14 P_i → Ala + H_2O + 2 CO_2 + 14 ATP

2. Ala + acetyl-CoA + NADH + H^+ → Pro + H_2O + NAD^+ + CoAH

The tsetse fly and the Colorado potato beetle belong to different orders within the Insecta and the question arises whether these are special metabolic cases within their respective groups. Because dipterans usually power their flight by the breakdown of carbohydrates (Sacktor, 1975) this seems likely for the tsetse fly. However, results of various investigations on beetles indicate that this feature may be more common in the order Coleoptera (see, for example, Crabtree and Newsholme, 1970; Pearson *et al.*, 1979).

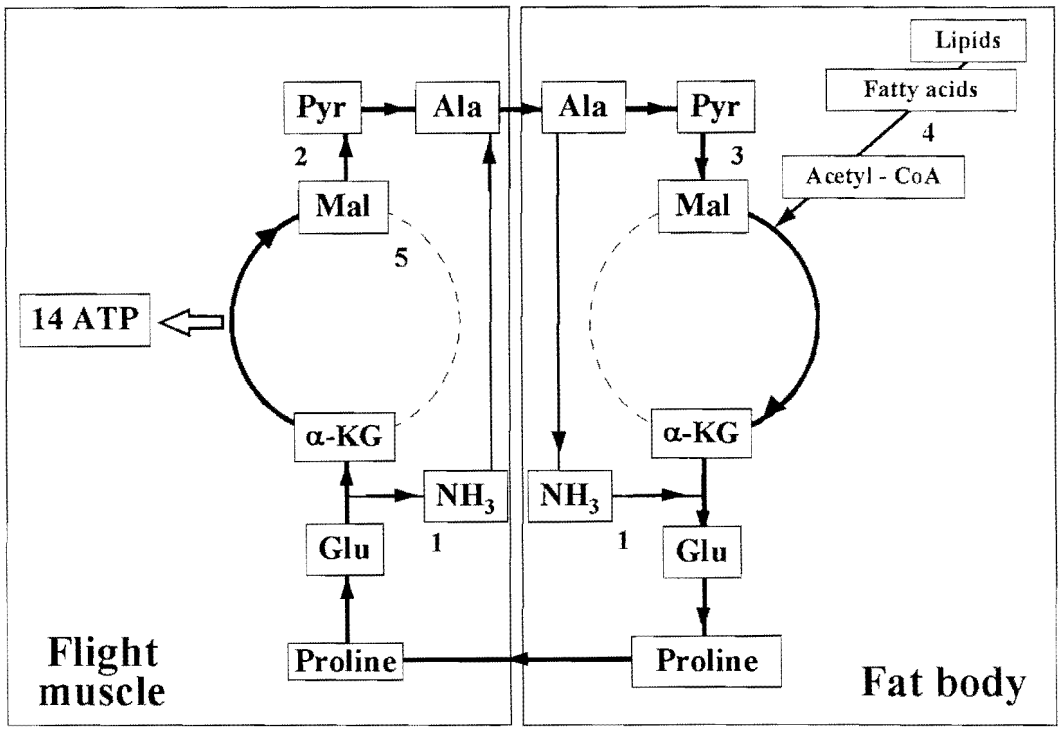

Figure 1. Biochemical pathways during proline oxidation in the flight muscles and re-synthesis in the fat body (adapted from Weeda *et al.*, 1980b). 1 = alanine aminotransferase, 2 = NAD-dependent malic enzyme, 3 = NADP-dependent malic enzyme, 4 = 3-hydroxyacyl-CoA dehydrogenase, 5 = malate dehydrogenase.

1.5. Scope of the study

The aim of this study was to provide a detailed examination of the flight metabolism in the fruit beetle, *P. sinuata*. Energy substrates used to power flight and mechanisms controlling the oxidation and synthesis of these substrates were examined along with the supply pattern and breakdown of the fuels. The extent to which these fuels participate in metabolism was quantified. The emphasis of the study was on the fate of proline, one of the major fuels in the fruit beetle.

Given the importance of flight muscle temperature for flight performance and the metabolic changes that occur during endothermic pre-flight warm-up in beetles, further investigation was undertaken to determine the energy source for this type of metabolism.

The role of the endogenous AKH peptide of *P. sinuata*, Mem-CC, in control of proline synthesis in *P. sinuata* was also examined. This included an investigation of the pattern of influence of Mem-CC on the haemolymph concentrations of proline and alanine, as well as the structural requirements for receptor recognition. Furthermore, novel neuropeptides were isolated from the corpora cardiaca of *P. sinuata* and other cetoniid beetles and their structure analysed to determine the involvement of these peptides in the regulation of metabolic processes.

Comparative experiments were performed using other beetle and insect species to elucidate additional information about the general role of proline as a fuel for flight in the Insecta.

2. MATERIALS AND METHODS

2.1. Experimental insects

2.1.1. Fruit beetles

Fruit beetles, *Pachnoda sinuata flaviventris* (Gory & Percheron; Scarabaeidae: Cetoniinae), were caught throughout the year (except July) in the vicinity of Cape Town, where they are common on roses, flowers of *Hibiscus* spp. and acacias, and a variety of other plants. They were kept in a constant-temperature room at 25 ± 2 °C with a relative humidity of 50 % and a day:night cycle of 14:10 h.

In addition to the captured animals, a captive colony provided animals at a rate of about 5–10 beetles per day. Between 200 and 300 adult fruit beetles were housed in each of two cages (35 cm x 45 cm x 45 cm) above a plastic tray (10 cm deep) that was filled with compost soil for egg laying. The soil was always kept moist to ensure hatching of eggs. The tray was exchanged once every two weeks, and eggs and larvae, together with the soil, were transferred into a wooden box of 90 cm x 65 cm x 60 cm that was filled to about 40 cm with compost soil. Larvae hatched after about 1 week and were then fed decaying fruit which was placed about 20 cm deep in the centre of the box. Larvae developed through their 3 instars (Holm and Marais, 1992) in this box, pupating after approximately 3 months. Beetles emerged after a further 5-8 weeks, and were collected daily and transferred into a separate cage. Adult beetles were fed with fresh fruit (bananas, guavas, peaches, pears and apples) *ad libitum* and sprayed with water every day.

Male beetles with an average weight of around 1000 mg (range from 630 to 1350 mg) were used in all experiments.

2.1.2. Blister beetles

Blister beetles, *Decapotoma lunata* (Pallas), of both sexes were caught on the slopes of Table Mountain, Cape Town, during the austral summer. Their average mass was 198 ± 76 mg (mean ± S.D., n = 17). Pilot experiments (see section 3.1.4.) showed that metabolite levels were affected by handling and the availability of food. Therefore, beetles were collected 1 day before experimentation and kept overnight with their food plant, flowers of *Bolusafra bituminosa* (L.) Kuntze (Fabaceae), in the insectary under the

conditions described in section 2.1.1.

2.1.3. Locusts

Two week old male migratory locusts, *Locusta migratoria,* were taken from a captive colony. The locusts were kept under crowded conditions at a temperature of 32 ± 2 °C, a relative humidity of 50 % and a light:dark cycle of 12:12 h. The animals were fed twice daily with fresh grass, supplemented with rolled oats or Pronutro®.

2.1.4. Cockroaches

Adult male cockroaches, *Periplaneta americana,* were obtained from a captive colony which was maintained under crowded conditions at a temperature of 28°C, a relative humidity of 60 % and a light:dark cycle of 14:10 h. The animals were fed with a mixture of dog food and Pronutro® and provided with water *ad libitum.*

2.1.5. Other insects

Several beetles species, *Trichostetha fascicularis, T. albopicta* (Scarabaeidae: Cetoniinae), and *Lepithrix* sp. (Scarabaeidae: Melolonthinae), were collected during the austral summer from protea flowers in the vicinity of Cape Town . *Dischista cincta, D. rufa* and *Leucocelis amethystina* (Scarabaeidae: Cetoniinae) were collected from acacia flowers on the Avon Heights farm near Somerset East, Eastern Cape. Cockchafers, *Melolontha melolontha* (Scarabaeidae: Melolonthinae), were collected near Heidelberg, Germany. Specimens of *Camenta innocua* (Scarabaeidae: Melolonthinae) were collected in Cape Town, where they flew into illuminated rooms after sunset in mid-summer. Carpenter bees, *Xylocopa capitata,* were caught at Kirstenbosch Botanical Gardens, Cape Town, where they were foraging on flowers of *Virgilia divaricata.* Caterpillars of the moth *Hippotion eson* (Sphingidae) were collected from leaves of the arum lily, *Zantedeschia aethiopica,* in Cape Town in the austral winter and reared on arum lily leaves to the adult stage in the laboratory at ± 22 °C. Cicadas, *Platypleura capensis,* were caught near Grabouw in the Western Cape during the austral summer and adult buprestid beetles were caught in the Karoo, South Africa. Mealworm beetles, *Tenebrio molitor,* were taken from a captive colony. They were kept in plastic containers on bran covered with cotton wool at 25 °C. Once a week the animals were provided with slices

of fresh potatoes.

2.2. Flight experiments

2.2.1. Cetoniid beetles

Experiments were performed at 24-25 °C in the laboratory, close to a window or a light source. Only beetles which started flight voluntarily and were absolutely quiet before the start of the experiment, i.e. did not walk around or open the elytra, were used.

At rest, and immediately after flight or warm-up (for definition see section 2.3.), 1µl haemolymph samples were taken from the intersegmental membrane between head and prothorax for each of the metabolite determinations (see below). In addition, haemolymph was sampled after various times of recovery following flight.

Beetles were dissected immediately after flight, or after certain periods of recovery. The head, prothorax, legs and wings were cut off, the gut was removed and the flight muscles were dissected. Subsequently, they were wrapped in aluminium foil and frozen in liquid nitrogen. Alternatively, the legless and wingless thorax was wrapped in aluminium foil and immediately frozen in liquid nitrogen. After removal of the gut, reproductive organs and Malphigian tubules from the abdomen, tracheae and airsacs with the surrounding fat body tissue were dissected and stored in aluminium foil as described above. Dissection of flight muscles and fat body of a beetle took approximately two minutes.

For investigation of the different flight muscle types (BM, DVM, DLM; for abbreviation see Fig. 2) of *P. sinuata*, whole thoraxes were frozen (see above). By hitting the frozen sample with a pestle in a grinder cooled by liquid N_2 the thorax fell apart and the different muscles (Fig. 2) were separated and treated separately further on.

2.2.1.1. Lift generating flight

Although this method describes a kind of tethered flight, it allowed the beetles to create lift as during natural free flight. This is in contrast to the flight method described in the following section.

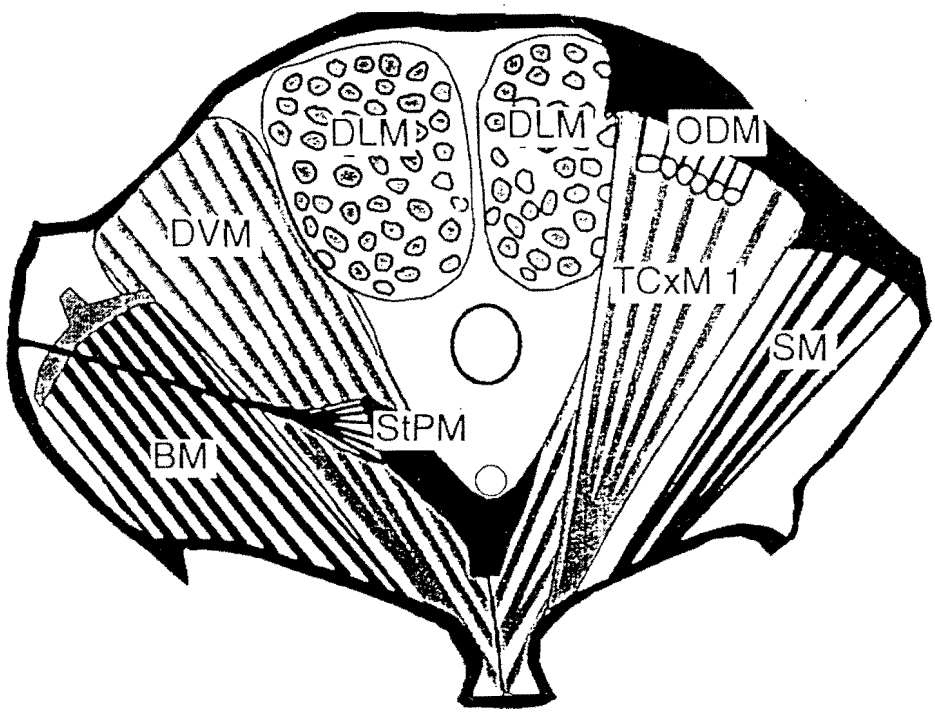

Figure 2. Flight muscles of *P. simulata* as seen from the front of the metathorax: BM = basalar muscle, DVM = dorso-ventral muscle, DLM = dorso-longitudinal muscle, SM = subalar muscle, ODM = oblique dorsal muscle, StPM = sternopleural muscle and TCxM 1 = tergocoxal muscle 1. (drawing by P. Schneider/Heidelberg).

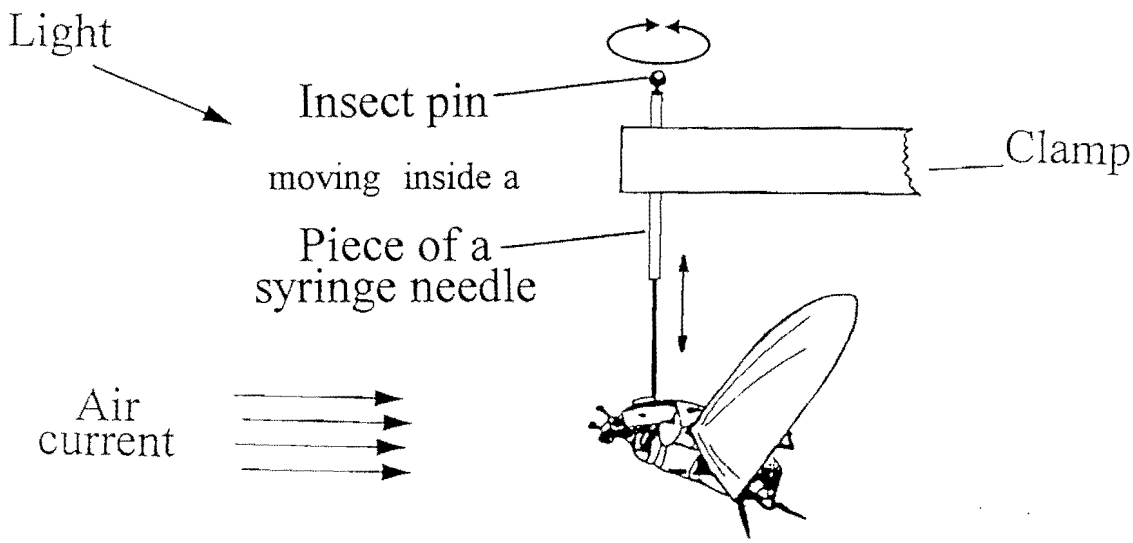

Figure 3. For lift generating flight the beetle was attached to an insect pin by means of dental wax. The insect pin was mounted in such a way that it could move inside a piece of a syringe needle. This allowed the beetle to move on their vertical and horizontal axis as indicated by arrows. The piece of syringe needle was held by a clamp (according to Schneider, 1989).

Beetles were attached to an insect pin at the prothorax by means of dental wax. The pin (weight: 180 mg) could move inside a piece of a syringe needle in such a way that it was possible to detect whether the animal produced lift (see Fig. 3). When the top of the pin moved up the insect was considered to produce lift. This method allowed the insects to turn around their vertical axis and move on their horizontal axis (Fig. 3). Animals which flew without continuously producing lift were excluded from the experiment. Before they started flying, beetles were allowed to move a ball of styrofoam with their legs. The animals were also provided with a frontal air current of 1.6 m/s.

This method was also used for flight experiments using carpenter bees.

2.2.1.2. Roundabout flight

Fruit beetles were fixed at the pronotum to a pipette tip with super glue. The pipette tip was then attached to an arm (length 29.5 cm) of a flight mill. With this flight method the beetles could fly forward, however, lift was minimised. Flight velocity was recorded by means of a light beam microprocessor tachometer (RS).

2.2.2. Flight experiments with blister beetles

A pilot experiment showed that carbohydrate levels decreased by about 35 % overnight, probably due to starvation. Feeding the captured beetles helped to keep carbohydrate levels higher but they were still 25 % lower than in newly captured beetles. For all further experiments, the beetles were kept as described in section 2.1.2. and all results shown in section 3.1.4. were obtained from those beetles.

Experiments were performed at 28-32°C in direct sunlight. Blister beetles were fixed to a thin thread between the head and prothorax. The thread was held by hand. This allowed the beetles to exhibit lift generating flight. Although individuals occasionally flew for longer than 30 min (maximum 58), most beetles were exhausted after 17 min. Exhaustion was recognised when the wings were extended, but no longer moving. For recovery experiments, beetles were flown for 10 min and subsequently rested in the dark without water and food for various periods.

Before and immediately after flight, and after various times of recovery following flight, 1µl haemolymph samples were taken from the neck membrane for each of the metabolite determinations (see below). Beetles that showed reflex bleeding during

handling (Nicolson, 1994) were discarded.

Immediately after flight, or after certain periods of recovery, beetles were dissected. The head, prothorax, legs, wings and abdomen were cut off and the gut was subsequently removed. The remaining whole thorax, which represents 24.0 ± 2.1 % (n = 5) of the total body mass, was wrapped in aluminium foil and immediately frozen in liquid nitrogen. The whole procedure required approximately 90 seconds.

2.3. Warm-up experiments with *P. sinuata*

To measure the metabolic changes during endothermic warm-up samples were taken of a beetle at rest and at its initial attempt to fly. This period included the warm-up and it was assumed that the majority of metabolic changes was caused by this process.

2.3.1. Thoracic temperature

In experiments where thoracic temperature (T_{th}) was measured during pre-flight warm-up and flight, a copper-constantan thermocouple (accuracy 0.2 °C), mounted in a syringe needle, was introduced laterally through a pre-drilled hole in the ventral part of the mesothorax into the metathorax. During measurements in the field, the thermocouple was pushed into the flight muscles immediately after capture.

2.3.2. Flight and warm-up experiments

Experiments were performed at 24 ± 2 °C in the laboratory close to a window, which was exposed to bright sunlight. For biochemical investigations the pin method described in 2.2.1. was used to allow the beetles to generate lift.

After the beetles were attached to the pin, they were sitting quietly until they started to warm-up. In most of the beetles, the beginning of warm-up was visible by the pumping movement of the abdomen, following which the head would also start to move back and forth in rhythm with the abdomen and, thereafter, the beetle would start to walk on the styrofoam ball. When the beetle would try to open the elytra to take off it would be interrupted for sample taking.

Before beetles were attached to the pin and immediately after flight or warm-up, 1µl haemolymph samples were taken from the intersegmental membrane between head and prothorax for each of the metabolite determinations. Immediately after flight or

warm-up, beetles were dissected. The head, prothorax, legs and wings were cut off, and the gut was subsequently removed. The flight muscle tissue was wrapped in aluminium foil and immediately frozen in liquid nitrogen. The whole procedure took not more than one minute. Preparation of samples and metabolite determinations were done as described in section 2.6.

2.3.3. Flight kinematics

For measurement of the following parameter, fixed flight was applied.

Wing beat frequency: The wing beat frequency was determined using an infrared light barrier which was placed at 90 ° to the plane of the wing beat and the ensuing impulses were recorded with an analogue electronic counter of own production.

2.3.4. Determination of respiratory quotient (RQ) and oxygen consumption

An open flow-through system was used. Change of oxygen concentration in the system was determined by a S-3A oxygen analyser (Applied Electrochemistry Inc.) fitted with a N-22M oxygen sensor and a R-1 flow control. The change of CO_2 concentration was measured with a 225 MK3 infrared gas analyser (ADC). Flow rate of the whole system was 250 ml/min for resting animals, 500 ml/min during fixed flight and warm-up and 600 to 700 ml/min during lift generating flight. The chambers had the following volumes; rest: 10 ml; warm-up: 50 ml; fixed flight: 265 ml and lift generating flight: 144 ml (Fig. 4). Thus, replacement times of one chamber volume was 2.5, 6.0, 32 or 12.3-14.4 s, respectively. These small chamber volumes and the high flow rates assured a quick response of the instruments so that correction methods for gas mixing could be omitted. Flow rate was measured by means of a burette filled with a solution of dish washing detergent (bubble flow meter) immediately after each sample. The oxygen analyser was connected behind the pump followed by the CO_2 analyser (Fig. 5). Air was pulled through the system. Before air reached the chamber it flowed through a container filled with Carbosorb (BDH) to remove atmospheric CO_2 and was dried by means of a plastic syringe filled with silica gel (BDH). A very narrow plastic pipe of 10 cm length containing silica gel was attached behind the chamber so that moisture, generated by the beetles, could be removed. The oxygen analyser was calibrated with atmospheric air and the zero value was checked with an oxygen-free gas mixture (CO_2 + N_2). The carbon

dioxide analyser was set to zero by filling the reference cell with either CO_2-free air, 350, 750 or 1450 ppm CO_2 (in N_2, oxygen-free) and calibrated with either 350, 750, 1450 or 2100 ppm CO_2 (in N_2, oxygen-free). All gas mixtures were from Afrox. Oxygen and carbon dioxide content was measured to the nearest 0.001%. The delay between the two analysers was 5-10 s. The system was equilibrated after inserting an animal into the chamber. Only then were measurements taken. Data obtained from animals which were immediately active in the chamber were discarded. The incoming air current in the flight chambers was directed to the beetles' heads. For measurement of the resting value, beetles were inserted into the respiration chamber and kept in the dark prior to measurement. Eight resting animals were used at a time. Beetle weight was determined immediately after each measurement.

These data, together with the measured flow rate, were used to calculate oxygen consumption per gram and hour at standard temperature and pressure (STP). Experiments were executed in a controlled temperature room at 28°C.

When only oxygen consumption was measured, the same system was used as described above, but the carbon dioxide analyser was omitted. The flow rate was set to 250 ml/min.

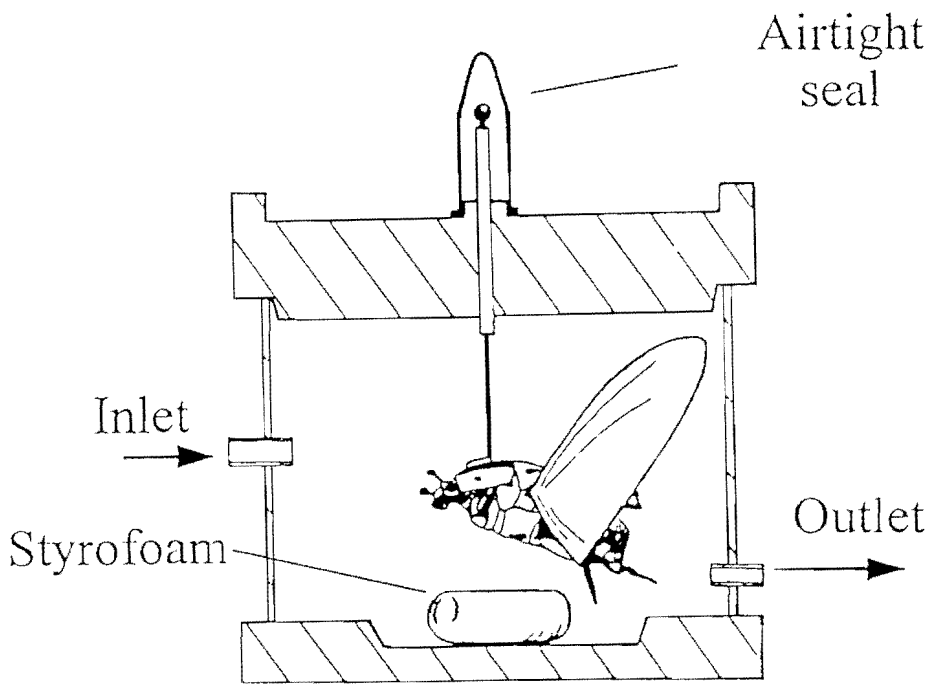

Figure 4. Respiration chamber for investigation of oxygen consumption and RQ during lift generating flight of *P. sinuata*. For details of attachment of the beetle see previous figure.

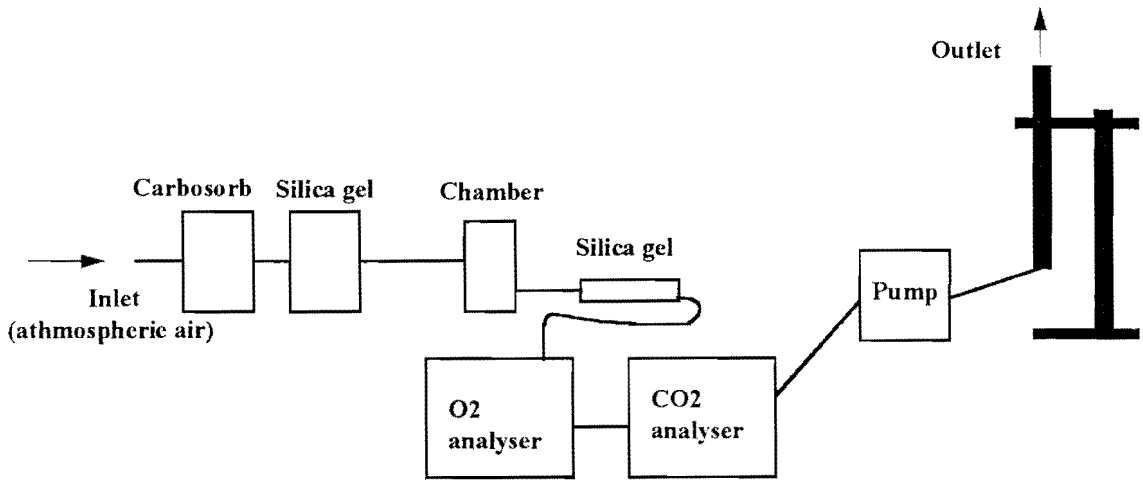

Figure 5. Experimental setup for RQ measurement. For measurement of oxygen consumption, CO_2 analyser was omitted. For details see section 2.3.4.

2.4. Starvation experiments

Groups of 10 adult males of *P. sinuata* were kept under the conditions described in section 2.1.1. in each of 10 glass containers (n = 100) without food but with water *ad libitum*. Faeces and dead beetles were removed daily from the containers to prevent the other individuals from feeding on them. Towards the end of the starvation period of up to 31 days, mortality was increasing but did not exceed 30 % in total. Beetles were fed with fresh guavas after 31 days. For determination of proline, alanine, lipids and carbohydrates, 1 µl haemolymph samples were taken before and at various times of starvation. For determination of energy substrates in flight muscles and fat body, beetles were killed at different times of starvation and samples were taken and treated as described in section 2.6.

Beetles were always taken randomly from different containers for an experiment. For bioassays during the starvation period, beetles from the same pool were used for all three sets of bioassays. Bioassays were carried out as described in section 2.10. The flight method described in section 2.2.1.1. was used for flight experiments during the starvation period.

2.5. Determination of haemolymph volume

A procedure similar to that of Clegg and Evans (1961) was used. In brief, a trace amount of (^{3}H-G) inulin, dissolved in 5 µl of water (37918 cpm), was injected ventrally into the abdomen between the last two segments with a 10 µl Hamilton syringe. After 20

min of incubation, a 1 µl sample of haemolymph was taken, pipetted into a scintillation vial containing 4 ml of scintillation fluid (Packard, Ultima Gold TR) and the radioactivity measured using a Tri Carb 460 instrument (Packard). The control was a blood sample of an uninjected beetle. The blood volume was calculated as follows:

$$V = \frac{\text{(cpm of injectate) x (volume of blood sample)}}{\text{(cpm of blood sample)}}$$

2.6. Determination of metabolite concentrations

2.6.1. Metabolites in the haemolymph

One µl of haemolymph was either blown immediately into 100 µl of concentrated H_2SO_4 for the determination of total lipids (Zöllner and Kirsch, 1962) or carbohydrates (Spik and Montreuil, 1964) or pipetted into 60 µl of 80 % acetonitrile for amino acid analysis (see section 2.6.3.).

2.6.2. Metabolites in flight muscle and fat body tissue

Preparation of extracts. Perchloric acid extracts from frozen tissues were made according to Zebe and Gäde (1993). Frozen tissue samples were ground to a fine powder in a mortar cooled by liquid N_2. The powder was then transferred into a pre-weighed Eppendorf tube containing perchloric acid (PCA, 1 M) which was adjusted to a tissue/PCA ratio of 1:5. The mixture was sonicated twice for 10 s by means of a Branson sonifier B 30 and centrifuged for 10 min at 8000 g (RC5C, Sorvall) at 4 °C. The pellet was re-suspended in PCA and the procedure repeated. The combined supernatants were neutralised by adding K_2CO_3 (3 M), the precipitated $KClO_4$ spun down (10 min at 8000 g, 4 °C) and the final volume of the extract measured. Extracts were stored at –20 °C until further use.

2.6.3. Analysis of metabolite concentrations

Total carbohydrates. Two ml of anthrone reagent (1.2 g anthrone in 300 ml distilled H_2O and 600 ml H_2SO_4) was added to the sample collected in 100 µl H_2SO_4, and then mixed by means of a vortex mixer and heated to 100 °C for 8 minutes. The sample was then cooled to room temperature in a water bath, incubated in the dark for 30 minutes, and the absorbence measured at 585 nm using a Vitatron IFP photometer.

Glucose was used as a standard.

Total lipids. The sample, which was blown into 100 µl of H_2SO_4, was mixed thoroughly, heated to 100 °C for 10 min and cooled to room temperature. One ml vanillin reagent (1.98 mg vanillin in 668 ml concentrated H_3PO_4 and 332 ml distilled H_2O) was added, mixed well, and incubated in the dark for 30 min. The absorbance at 546 nm was measured by means of a Vitatron photometer with cholesterol as a standard.

Glycogen determination. Glycogen was precipitated from an aliquot of PCA extract with 5 ml ethanol, to which 200 µl Na_2SO_4 (saturated) was added. The mixture was kept refrigerated overnight and centrifuged for 20 min at 10000 g at 4 °C. The precipitate was re-dissolved in 1 ml water and 25 µl samples were analysed by the anthrone method (see 2.6.3.) with glucose as a standard.

Amino acid determination. Derivatisation of 25 µl aliquots of PCA extracts or haemolymph sample took place by adding 100 µl $NaHCO_3$ (0.5 M, pH 8.5) and 100 µl dansylchloride (6 mg/ml acetone). The mixture was incubated in the dark for 3 h at room temperature. Incubation was stopped by adding 800 µl $NaHCO_3$ solution. Quantification of the derivatised amino acids was achieved by means of a Beckman HPLC System Gold equipped with a solvent module 126, detector module 166, an autosampler module 507, a module 406 analogue interface and an IBM-compatible Samsung computer SD 620. Peak areas were integrated using the Beckman System Gold software. Separation was achieved using a 125 x 5 mm Spherisorb ODS II column (5 µm material) equipped with a 20 mm guard column of the same material (Bischoff Chromatography).

The HPLC buffers were: (A) formic acid (50 mM), acetic acid (60 mM); (B) 35 % 2-propanol, in solvent A. A linear gradient of 25 to 80 % solvent B over 25 min was applied at a flow rate of 1 ml/min. The absorbance was measured at 254 nm. Standard solutions of proline, alanine, aspartate and glutamate were used to identify and quantify the correct peaks.

2.7. Determination of maximum enzyme activities

2.7.1. Preparation of tissue

Fruit beetles, blister beetles and locusts were kept at 4 °C in the dark 30 min prior to dissection. The fruit beetle's head, pro- and mesothorax were cut off, and abdomen and thorax separated. Flight muscles and, after removal of the gut, reproductive organs, Malphigian tubules and fat body tissue were dissected and transferred into ice-cold homogenisation buffer, which consisted of TRA/HCl (100 mM), and EDTA (100 mM), pH 7.6. The procedure for locusts was similar, except that the gut was pulled out with the head. Whole thoraces of blister beetles were used after removal of head, gut and abdomen. Tissues were rinsed twice, with the above mentioned buffer, homogenised by means of an Ultra Turrax at maximum speed for 2 x 10 s and subsequently sonified using a Branson B 30 sonifier for 10 s. The homogenate was centrifuged at 8000 g and 4 °C for 20 minutes and the supernatant used for all the assays, with the exception phosphorylase. For this enzyme, tissues were homogenised in a buffer consisting of TRA/HCl (50 mM, pH 7.0), EDTA (5 mM), NaF (20 mM).

2.7.2. Enzyme assays

All determinations were carried out at 340 nm and 25 °C in 2 ml cuvettes in a LKB Ultrospec spectrophotometer. The final volume was 1 ml. Determinations were at least performed in duplicate, with different concentrations of extract used to ensure linearity. The various enzyme activities were measured under the following conditions:

Glutamate dehydrogenase (EC 1.4.1.2.): TRA/HCl (80 mM), EDTA (8 mM), $(NH_4)_2SO_4$ (55 mM), NADH (0.3 mM), ADP (2.5 mM), pH 7.6. The reaction was started with α-ketoglutarate (4 mM).

3-hydroxyacyl-CoA dehydrogenase (EC 1.1.1.35): TRA/HCl (100 mM, pH 7.0), EDTA (5 mM), NADH (0.3 mM). The reaction was started with acetoacetyl-CoA (0.4 mM).

NAD-dependent malic enzyme (EC 1.1.1.39): TRA/HCl (50 mM, pH 7.8), EDTA (5 mM), $MgSO_4$ (8 mM), NAD (0.5 mM), malate dehydrogenase (2.5 U/ml). The reaction was initiated with L-malate (2 mM) after malate dehydrogenase equilibrium.

NADP-dependent malic enzyme (EC 1.1.1.40.): TRA/HCl (50 mM, pH 7.8), EDTA (5 mM), $MgSO_4$ (8 mM), NADP (0.5 mM). The reaction was started with L-

malate (2 mM).

Malate dehydrogenase (EC 1.1.1.37): TRA/HCl (50 mM, pH 9.8), NAD (2 mM), $MgSO_4$ (5 mM). The assay was started with L-malate (10 mM).

Alanine aminotransferase (EC 2.6.1.1.): TRA/HCl (50 mM, pH 7.4), L-alanine (50 mM), NADH (0.3 mM), lactate dehydrogenase (5 U/ml). The reaction was initiated with a-ketoglutarate (4 mM).

Aspartate aminotransferase (EC 2.6.1.2.): TRA/HCl (50 mM, pH 7.4), L-aspartate (40 mM), NADH (0.15 mM), malate dehydrogenase (0.63 U/ml). The reaction was started with a-ketoglutarate (7 mM).

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (EC 1.2.12.): TRA/HCl (50 mM, pH 7.6), ATP (1.5 mM), EDTA (5 mM), NADH (0.15 mM), $MgSO_4$ (3.3 mM), mercaptoethanol (2.4 mM), 3-phosphoglycerokinase (200 U/ml). The reaction was started by addition of 3-phosphoglycerate.

For determination of *glycogen phosphorylase* (EC 2.4.1.1.) in the direction of glycogen breakdown, the medium contained TRA/HCl (50 mM, pH 7.0), 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/ml), glucose-6-phosphate dehydrogenase (0.85 U/ml). The reaction was started with the addition of extract.

2.8. Respiration of flight muscle mitochondria

2.8.1. Isolation of flight muscle mitochondria

The method used was adapted from Slack and Bursell (1976) and Weeda *et al.*, (1980a). Throughout isolation the temperature was kept at 4 °C. Flight muscles of two cetoniid beetles were dissected, chopped into small pieces with scissors and transferred into an isolation medium containing phosphate buffer (15 mM), EDTA (1 mM), Nagarse protease (1 mg/3 ml medium), with pH 7.2. The sample was then incubated for 5 min under constant agitation with a magnetic stirrer bar of about 1 cm, poured through a sieve (0.3 mm mesh), the filtrate was then layered on top of 5 ml of isolation medium containing sucrose (250 mM) in a centrifuge tube and centrifuged at 3500 g for 5 min. Supernatant was then decanted and the top layer of the pellet washed with isolation medium. The pellet was then re-suspended in storage medium containing sucrose (320

mM), phosphate buffer (5 mM), EDTA (1 mM), pH 7.2 and kept in ice-cold water until use (latest 2h after preparation).

Flight muscles of two locusts provided sufficient material, whereas each blister beetle or monkey beetle preparation was made from 10 or 8 whole thoraces, respectively.

2.8.2. Determination of respiration rate of isolated mitochondria

Respiratory rates of isolated mitochondria were measured polarographically at 25 °C in a final volume of 2 ml of an incubation medium containing KCl (120 mM), phosphate buffer (5 mM), sucrose (40 mM) and BSA (1 %, essentially fatty acid free) by means of the YSI model 5300 oxygen monitoring system. Data were recorded as percentage of oxygen saturation, using air-saturated distilled water and an oxygen free Na_2SO_3 solution as references.

Substrate preference and co-oxidation as well as the substrate dependence of proline and pyruvate oxidation were measured during state 3 respiration, thus, in the presence of ADP (respiration states according to Chance and Williams, 1956). For estimation of the intactness of the mitochondria after isolation, the respiratory control ratio (RCR) was calculated. This is the ratio of ADP stimulated respiration (state 3) to the resting rate in the absence of ADP (state 4) (Estabrook, 1967). Another measure applied in the present study for the intactness of the mitochondria was the state of coupling of oxidative phosphorylation to electron transport through the respiratory chain. This is expressed in the ADP:O ratio, the rate of disappearance of P_i versus oxygen uptake (Estabrook, 1967).

ADP as well as the appropriate substrates and substances were added in a volume not exceeding 2 % of the final volume. Palmitoyl-carnitine was added as an ethanolic suspension (20% ethanol). For this test, ethanol (20 %) was used as control.

Protein was measured according to Bradford (1976) using a BioRAD® kit and bovine serum albumin (fraction V) as a standard.

2.9. Proline synthesis of *P. sinuata* fat body *in vitro*

2.9.1. Preparation of fat body tissue

The fat body of *P. sinuata* is a thin layer of cells which surrounds the airsacs and tracheoles in the abdomen of the beetle. The cells are loosely attached to these organs. Under the light microscope they appear as a grey matter which is filled with a large amount of oil droplets. The cells are easily damaged, and the oil droplets are then released.

Male beetles up to 3 weeks old were taken from a captive colony. Before killing, beetles were kept in the refrigerator for 30 min. All the tools were cleaned prior to dissection with 96 % ethanol. The dorsal cuticle of the abdomen was cut off, and gut, reproductive organs and Malphigian tubules removed. The ventral part of the abdomen with the attached fat body was subsequently transferred and washed in ice-cold *Pachnoda* saline (modified after Nicolson, personal communication), which consisted of: NaCl (110 mM), KCl (25 mM), $CaCl_2$ (2 mM), $MgCl_2$ (5 mM), $NaHCO_3$ (10 mM), NaH_2PO_4 (5 mM); it was adjusted to pH 7.0 (with 0.5 N NaOH) and to 500 mOsm (with sucrose). Thereafter, pieces of abdomen were transferred into Eppendorf tubes containing fresh saline.

2.9.2. Incubation experiments

The ventral parts of the abdomen were pre-incubated for 2 x 2 hours at 35 °C in order to remove endogenous substrates. Before the second pre-incubation period the saline was changed. Thereafter, the abdomens were taken out and washed in saline. The fat body halves of each animal were dissected and transferred into separate, pre-weighed Eppendorf tubes containing 90 µl of saline with several additions, either labelled or unlabelled. The left half was always the experimental one and the right half the control, which was incubated in saline.

Incubation took place in a water bath at 35 °C under constant gentle shaking. This temperature was chosen according to abdominal temperatures measured in *P. sinuata* during flight (Heinrich and McClain, 1986). A 5 µl sample of each incubation was taken at time 0 and at several times during the incubation and pipetted into 100 µl of 80 % acetonitrile for amino acid determination. After termination of an experiment, the incubation vessels with the fat bodies and the incubation medium were frozen for protein

determination which was executed as described above (section 2.8.2.).

Amino acid determination was carried out as described in section 2.6.2. Radioactivity present in amino acid peaks were detected by means of a β-ram detector (INUS Systems) which was connected in series with the UV-detector of the Beckman HPLC system. Quicksafe Flow 2 (Zinsser Analytic) was used as scintillation fluid at a flow rate of 3 ml/min (ratio 1:3 to HPLC solvent).

2.9.3. Investigation of fat body lipids

The fatty acid content of the lipids in *P. sinuata* fat body tissue was analysed by the Fisheries Industry Research Institute, Cape Town, by gas chromatography. Extraction and analysis were performed according to de Koning *et al.* (1985). The instrument used was a 5710A Hewlett Packard gas chromatograph fitted with a 48 m glass capillary column (0.3 mm diameter) coated with Silar 10C. Hydrogen was used as carrier gas.

For thin layer chromatography (TLC) the tissue was homogenised in a chloroform-methanol mixture (2:1), using a Branson sonifier B 30, and the homogenate spun at 15000 g and 4 °C for 10 min. The supernatant was collected, and the lipids were extracted according to Folch *et al.* (1957). The radioactivity before separation as well as in the upper and lower phases after separation was detected using a Beckman LS 5000 TD scintillation counter. The radioactivity was calculated as cpm per mg protein of the tissue sample. The lower phase containing the lipid fraction was vacuum dried and kept at -20 °C until further use.

Thin layer chromatography. The method used was essentially that of Mangold (1965). The dried sample of the lower phase (see above) was re-dissolved in developing solvent which consisted of petroleum ether (bp 60-70 °C) : diethyl ether : acidic acid, 90:10:1 (v/v). Oleic acid, palmitic acid, monoolein, diolein and triolein, dissolved in developing solvent, were used as standards.

Samples and standards were applied to Whatman LHP-K plates (silica gel) using microcapillaries (Brand).

After application and drying of samples, the plates were developed in a chamber in which the walls were covered by Whatman No 1 filter paper soaked with developing solvent. The level of developing solvent in the chamber was about 5 mm.

After developing, plates were removed from the chamber, dried with a hairdryer and introduced into a chamber containing iodine. After 10 min, when the separated lipid fractions were stained brown, plates were removed from the chamber and the result recorded.

For autoradiography, the plates were exposed to X-ray films. Either Cronex 4 X-ray film or MPO-film were used in X-ray cassettes in the presence of an intensifying screen. Exposure was performed at room temperature. Exposure times ranged from 12 h to 1 week. The film was developed for 4 min, and fixed for 2 min.

The radioactive spots on the TLC plates, visible in the respective autoradiograms, were scratched out, transferred into scintillation vials containing 4 ml scintillation fluid (Packard, Ultima Gold TR) and the radioactivity measured using a Beckman LS 5000 TD scintillation counter. The radioactivity was calculated as cpm per mg protein of the tissue sample.

2.10. AKH peptides

2.10.1. Isolation of corpora cardiaca peptides

Preparation of extracts of corpora cardiaca. For dissection of corpora cardiaca, beetles were cooled in a refrigerator 30 min before use. The prothorax, with the head, was separated from the rest of the body. The head was cut open dorsally with a razor blade and the exposed corpora cardiaca could clearly be seen at 35-fold magnification. The glands were dissected into 80 % methanol and frozen at -20 °C. For extraction, glands were sonicated twice with a Branson B 30 sonifier and centrifuged for 3 min at 20120 g in a Heraeus Sepatech desk centrifuge. The supernatant was collected, and the procedure repeated with the pellet. The combined supernatants were dried by means of a vacuum concentrator (Bachofer). The extracts were either taken up in distilled water (for bioassays) or in 25 % HPLC solvent B (for separation on HPLC).

Purification of AKH peptides. Crude methanolic gland extracts were applied to a Nucleosil 100 C-18 column (4.6 x 125 mm, particle size 5 µm) equipped with a guard column (20 mm) of the same material (Macherey & Nagel), for reversed-phase liquid chromatography (RP-HPLC). A Gilson HPLC system was used, consisting of two model 302 piston pumps with a 5 SC pump head, a manometric model 802, a Model 811

mixing chamber, a Rheodyne Model 7125 sample injector with a 500 µl loop, and an LKB 2151 variable wavelength detector (10 µl HPLC flow cell, 10 mm path length). The pumps were controlled by a microcomputer Apple II+. A Shimadzu RF–535 monitor was used to detect the fluorescence signal (caused by Trp) of the AKH peptides at an excitation wavelength of 276 nm and an emission wavelength of 350 nm.

HPLC solvents: (A) was 0.11 % TFA and the composition of (B) was 60 % acetonitrile in 0.1 % TFA. Separation was achieved using a linear gradient from 43 to 53 % solvent B in 20 min at a flow rate of 1 ml/min. For better separations of closely eluting peptides, a gradient of 33 to 53 % B in 40 min at a flow rate of 1 ml/min was applied.

One minute fractions or peak fractions were collected manually and dried in a vacuum centrifuge.

2.10.2. Sequence determination and mass spectrometry

Removal of N–terminal pyroglutamate. Sequencing by Edman degradation uses the 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. Therefore, the pyroglutamate, which blocks the N–terminus of all peptides of the AKH/RPCH family, has to be removed. Purified (see above) peak material (representing the peptide) was enzymatically deblocked using L–pyroglutamate aminopeptidase (EC 4.4.19.3; sequencing grade, Boehringer Mannheim). The dried peptidic material was taken up in 60 µl buffer and 20 µl pyroglutamate aminopeptidase (2.88 mg/ml buffer). The buffer had the following composition: Na_2HPO_4 (100 mM), EDTA (10 mM), DTT (5 mM), glycerol (5 % v/v), pH 8.0. The mixture was incubated at 50 °C for 3 h and the reaction stopped by addition of an equal volume of a solution containing 15 % acetonitrile and 0.1 % TFA. The mixture was now applied to the above described HPLC system; the gradient ran from 33 to 53 % solvent B in 40 min at a flow rate of 1 ml/min. Peak material was collected manually and dried in a vacuum centrifuge.

Edman sequencing. The deblocked peptide was sent to Dr. R. Kellner (University of Mainz, Germany). 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.

Mass spectrometry. Dr. R. Kellner (University of Mainz, Germany) and Dr. R. Predel (Texas A & M University, USA) investigated the mass of the peptides by using matrix-assisted laser desorption ionisation (MALDI) time of flight (TOF) mass spectrometry.

2.10.3. Bioassays

Bioassays in beetles. Bioassays with the different beetle species were performed as follows. Beetles were kept separately in small plastic containers the night before experimentation at room temperature (± 22 °C). Before and 90 min after injection of the appropriate test solution, a 1 µl haemolymph sample was taken from the neck membrane and used for the determination of total carbohydrates, total lipids or for amino acid analysis.

Bioassays in locusts and cockroaches. Adult, male locusts were kept under funnels, and adult, male cockroaches in small containers for at least 2 h at room temperature before experimentation. A 1 µl sample was collected just before the animal was injected with the appropriate substance in a volume of 10 µl. A second sample was taken 90 min (locusts) or 120 min (cockroaches) later and the haemolymph lipids (locusts) or haemolymph carbohydrates (cockroaches) determined as in 2.6.2.

2.11. Chemicals, biochemicals and synthetic peptides

2.11.1. Synthetic peptides

Mem CC, Del-CC, Ona-CC, Scd-CC-I, Scd-CC-II, Tem-HrTH, Ani–AKH, Pht–HrTH, Lom-AKH–III and an analogue of Mem CC with Asn^7 instead of Asp^7 were synthesised by Dr. R. Kellner(University of Mainz, Germany). The peptides were purified by RP–HPLC and their identities verified by amino acid analysis and mass spectrometry. Synthetic Pea-CAH-I and Pea-CAH-II were obtained from Peninsula Laboratories (USA).

2.11.2. Radiochemicals

The universally carbon-labelled alanine, aspartate, proline, tyrosine and palmitic acid were purchased from ACE-Amersham. They had the following specific activities: (^{14}C-UL) proline, 252 mCi/mmol; (^{14}C-UL) alanine, 154 mCi/mmol; (^{14}C-UL) aspartate, 212 mCi/mmol; (^{14}C-UL) tyrosine, 423 mCi/mmol and (^{14}C-UL) palmitate, 829 mCi/mmol. (^{3}H-G) inulin was obtained from DuPont and had a specific activity of 355.3 mCi/g.

2.11.3. Enzymes

Protease type XXVII (Nagarse, EC 3.4.21.62) was purchased from Sigma; Phosphoglucomutase (EC 5.4.2.2), glucose-6-phosphate dehydrogenase (EC 1.1.1.49), 3-phosphoglycerokinase (EC 2.7.2.3), lactate dehydrogenase (EC 1.1.1.27), malate dehydrogenase (EC 1.1.1.37) and pyroglutamate aminopeptidase (EC 4.4.19.3) were obtained from Boehringer Mannheim.

2.11.4. Other chemicals

Other chemicals were of the highest available purity and obtained from a variety of sources, including Boehringer/Mannheim, Merck, Saarchem, BDH and Sigma.

2.12. Statistical analysis

For determination of significance, Student's t-test or, where appropriate, paired t-test was used. For significance levels and further details see figure legends.

3. RESULTS

3.1. Flight experiments

3.1.1. Lift generating flight of the fruit beetle, *Pachnoda sinuata*

3.1.1.1. Flight duration

The majority of beetles flew for between 1 and 5 min. Occasionally an individual beetle would fly for up to 20 min; but this occurred in less than 5 % of the sampled beetles (n = 42).

3.1.1.2. Concentrations of metabolites in the haemolymph

Resting values of proline and alanine in the haemolymph were 98 and 8 µmol/ml, respectively (Fig. 6A). Because significant metabolic changes occur during pre-flight warm-up in *P. sinuata* (see section 3.2.2.), metabolite concentrations were also measured after the beetles had completed this phase. At this time, the proline concentration had already dropped to 78 µmol/ml and that of alanine had risen to 30 µmol/ml. The proline concentration dropped further during 30 s of flight, levelling at around 50 µmol/ml. Concomitantly, the alanine concentration increased to 42 µmol/ml during 30 s of flight and to 62 µmol/ml after 5 min of flight (Fig. 6A).

During rest after a 5 min flight period (Fig. 6A), proline concentration increased while the opposite was observed in alanine with a decrease of concentration. However, the pre-flight levels of the two amino acids were not yet reached after 60 min of rest. The sum of the two amino acids was more or less stable during flight and subsequent rest, at approximately 110 µmol/ml, with the exception of the 90 µmol/ml recorded after 30 s of flight.

The concentration of haemolymph carbohydrates was about 7 mg/ml at rest before flight and was not influenced by warm-up. The concentration increased during 30 s of flight to around 9 mg/ml and later dropped consistently (Fig 6B). After 5 min of flight, less than 6 mg/ml was measured. Carbohydrate concentration rose to 7.5 mg/ml, i.e. reaching pre-flight levels during 60 min of rest following a 5 min flight period.

Haemolymph lipids showed a small but significant increase ($p < 0.001$) in 13 beetles which flew to exhaustion (11.0 ± 3.8 min), during which time lipid levels increased by 1.5 ± 1.1 mg/ml from an initial 10.0 ± 3.1 to 11.5 mg/ml. After 60 min of

subsequent rest, 14.0 ± 6.0 mg/ml of haemolymph lipids were measured.

For clarity the warm-up values are not depicted in Figures 6 and 7, but taken into account for calculations in Table 23.

3.1.1.3. Metabolite concentrations in flight muscles

The most dramatic changes in flight muscle metabolite concentrations occurred during the first 30 s of flight (Fig. 7A,B). During warm-up, proline concentrations dropped from the resting value of 55 to 47 µmol/gfw. During the first 30 s of flight, proline levels decreased further to 31 µmol/gfw. In contrast to proline levels in the haemolymph, proline levels in the flight muscles dropped further to reach 13 µmol/gfw after 5 min of flight.

In comparison, the resting alanine concentration of 11 µmol/gfw increased to 22 µmol/gfw during warm-up and rose further to 36 µmol/gfw during 30 s of flight (Fig. 7A). Again, in contrast to the haemolymph concentrations, alanine in the flight muscle continued to increase to 55 µmol/gfw after 5 min of flight. Pre-flight levels of proline and alanine were re-established after one hour of rest following a 5 min flight. The sum of the proline and alanine concentrations was in the range of 64 to 70 µmol/gfw during the whole experiment.

Glycogen stores in the flight muscles, amounting to 77 µmol glucose equivalents/gfw, were not influenced by pre-flight warm-up. The warm-up value of 72 µmol/gfw did not differ significantly from the resting value. The steepest decrease in glycogen concentration occurred within 10 s of flight after which time 48 µmol/gfw (Fig. 7B) were measured. Thereafter, glycogen levels were less dramatically affected with 12 µmol/gfw recorded after 5 min of flight while after 60 min of rest glycogen concentrations were almost back to pre-flight levels.

3.1.2. Flight without lift production of *Pachnoda sinuata*

3.1.2.1. Flight duration

In contrast to beetles in lift generating flight, those on the flight mill (n = 48) flew at least 45 min, with more than 10 % flying for 2 h. The longest flight was recorded at 2 h 45 min.

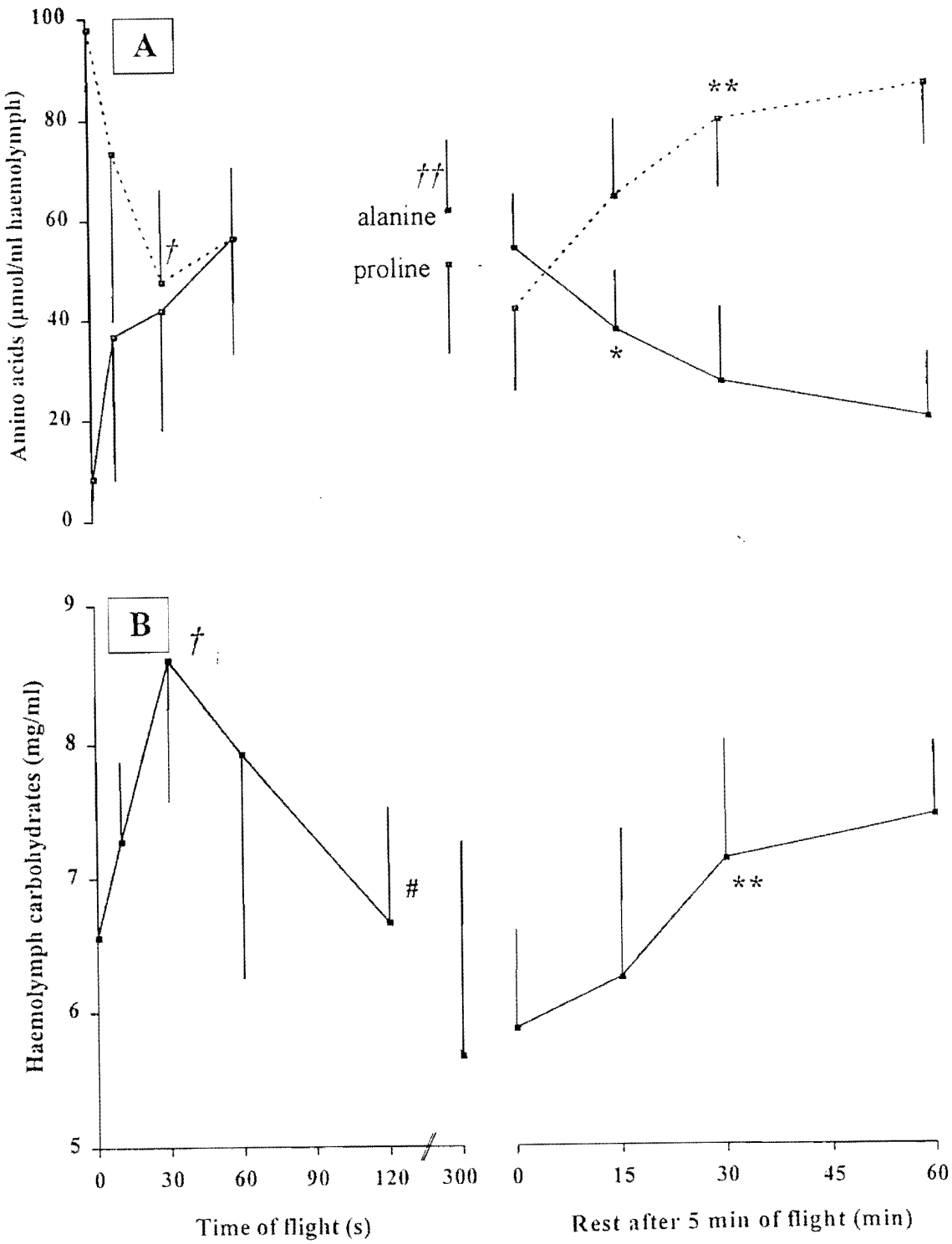

Figure 6. Metabolite concentrations in the haemolymph of *P. sinuata* during different durations of lift generating flight and subsequent rest after flight: A. Proline (dashed line) and alanine (solid line); B. Total carbohydrates. Values are given as means ± S.D. (n = 5-6). For clarity, significance is only depicted when the change first became significant in comparison with the reference value. In Fig 6A value after pre-flight warm-up was taken as the reference for animals which flew, in Fig 6B resting value was taken (no change during warm-up). Significance levels applied were $p < 0.05$ (†) and $p < 0.01$ (††). For the resting period following flight, values were compared with those when flight was terminated. Significance levels indicated are: $p < 0.05$ (*) and $p < 0.01$ (**). The change in Fig 6B was also compared with the value of 30 s of flight, after which concentration dropped, $p < 0.05$ (#).

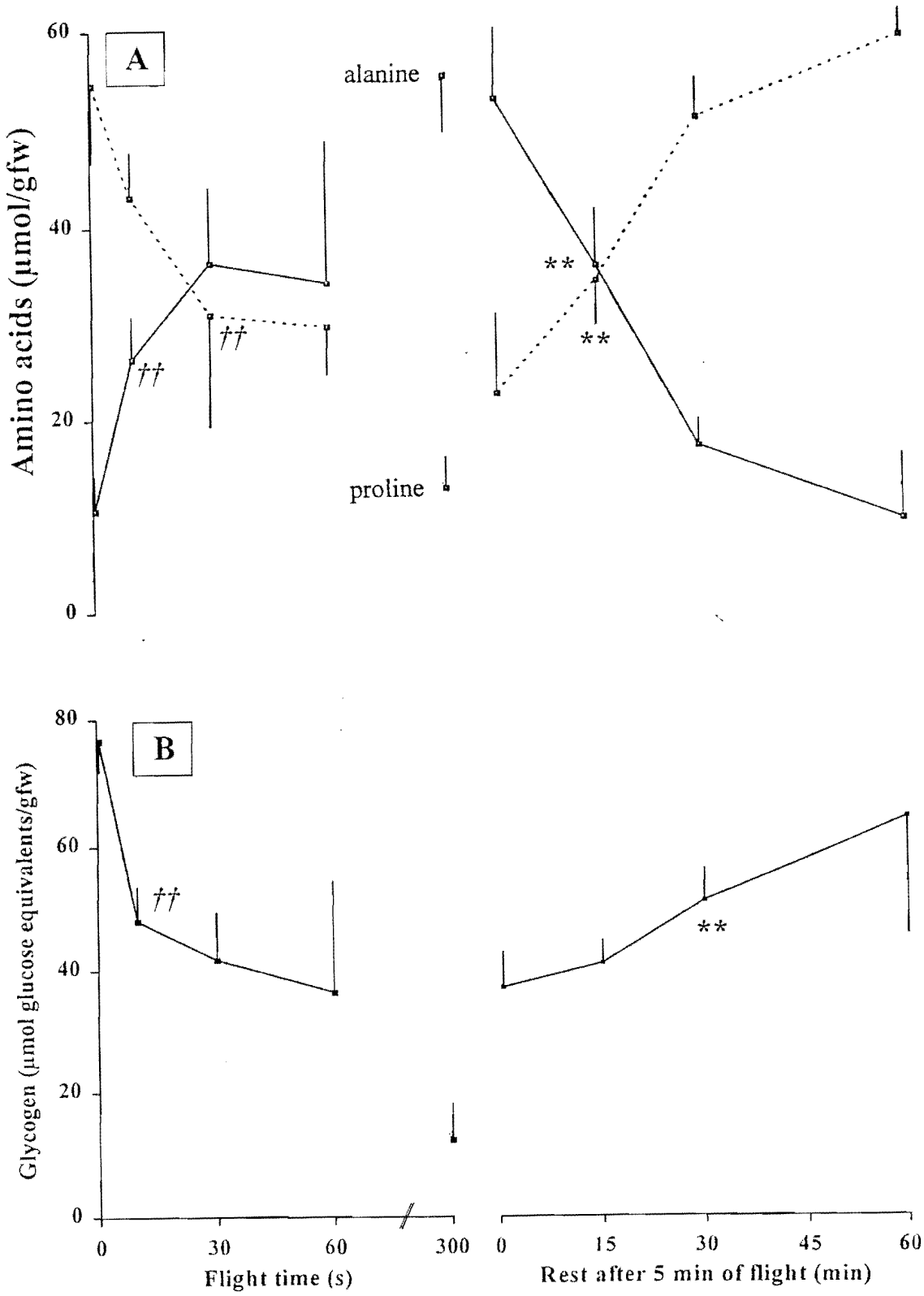

Figure 7. Metabolite concentrations in flight muscle tissue of the complete flight muscles of *P. simulata* during different durations of flight with lift and subsequent rest after 5 min of flight: A. Proline (dashed line) and alanine (solid line); B. Glycogen. Values are given as means ± S.D.; (n = 5 - 8). For significance levels see legend to Fig. 6. Reference value for beetles which flew was the warm-up value. For the resting period following flight, values were compared with those when flight was terminated.

3.1.2.2. Concentrations of metabolites in the haemolymph

The following concentrations of metabolites were found in resting beetles: proline 128.9 µmol/ml, alanine 14.1 µmol/ml, total carbohydrates 9.4 mg/ml and lipids 8.5 mg/ml (Fig. 8A,B,C).

The proline concentration in the haemolymph decreased during the first 15 min of flight to 40 µmol/ml when it reached a low plateau of 30-40 µmol/ml (Fig. 8A). In contrast, alanine concentrations rose to 74 µmol/ml after 15 min of flight and remained at a level of 90-100 µmol/ml in beetles which flew for 30 min or longer (Fig. 8A). Pre-flight proline levels were reached after 60 min of rest following a flight of 30 min, while pre-flight alanine values were already reached after 15 min of rest. The sum of the proline and alanine concentrations was more or less stable throughout the experiment (between 120 and 145 µmol/ml) with the exception of the value for 15 min of flight, which was only 114 µmol/ml. During subsequent rest the sum is smaller, ranging from 101 to 129 µmol/ml with the lowest value at 15 and 30 min after termination of flight.

The resting haemolymph concentration of aspartate was 0.47 ± 0.19 µmol/ml (n = 5) while that of glutamate was 0.65 ± 0.23 µmol/ml (n = 5). No changes were recorded in either of these two amino acids after 30 min of flight and 60 min of rest thereafter (data not depicted).

The level of total carbohydrates in the haemolymph dropped from an initial 9.4 to 4.8 mg/ml during 30 min of flight (Fig. 8B). Later, higher concentrations were measured, reaching pre-flight levels again after 90 min of flight. In beetles which flew for 30 min, pre-flight values of carbohydrates were only reached after 120 min of rest.

The haemolymph lipid concentration increased in an almost linear fashion during the first 60 min of flight, from 9.4 to 16.3 mg/ml. Concentrations subsequently remained at this level (Fig. 8C). In beetles that rested after 30 min of flight, lipid levels were not restored to pre-flight levels.

3.1.2.3. Metabolite concentrations in flight muscles

Flight muscles contain about 59 µmol/gfw proline and 5 µmol/gfw alanine at rest (Fig. 9A). During 15 min of flight proline concentration dropped to 22 µmol/gfw and remained at a level of about 20 µmol/gfw after longer flight periods (Fig. 9A). Concomitantly, alanine concentrations rose to 38 µmol/gfw during a flight of 15 min

reaching a plateau of more than 40 µmol/gfw after 30 min or longer. Pre-flight levels of the two amino acids were observed after 60 min rest following a 30 min flight. The sum of proline and alanine concentrations was very stable, between 60 and 65 µmol/gfw, throughout flight and subsequent rest.

The resting flight muscle concentration of aspartate was 0.42 ± 0.09 µmol/gfw (n = 5) while that of glutamate was 0.50 ± 0.18 µmol/gfw (n = 5). There were no changes observed in either of these two amino acids after 30 min of flight and 60 min of rest thereafter.

In flight muscles of resting beetles a glycogen concentration of about 65 µmol/gfw was measured (Fig. 9B). These stores were basically depleted during 15 min of flight (to 16 µmol/gfw; Fig. 9B). There was a further negligible decrease to about 10 µmol/gfw during the next 30 min of flight. Recovery of glycogen stores to pre-flight levels after 30 min of flight took about 120 min.

3.1.2.4. Metabolite concentrations in fat body

In the fat body of *P. sinuata* resting levels of proline are lower than in flight muscle tissue (43.2 µmol/gfw, Fig. 10A). Changes in proline during flight were less steep than those observed in flight muscles and haemolymph (Fig. 10A). In the first 15 min proline concentration decreased to 28 µmol/gfw, decreasing only slightly thereafter (Fig. 10A) with the final concentration after 45 min of flight 22 µmol/gfw. Similarly, there were smaller increases in alanine concentration than in flight muscles. From an initial 7 µmol/gfw, alanine concentrations increased to 21 µmol/gfw (Fig. 10A). During subsequent rest after 30 min of flight, proline and alanine concentrations returned to pre-flight levels within 120 min. Total concentrations of proline and alanine ranged from 44 to 50 µmol/gfw during flight and subsequent rest.

The concentration of aspartate in the fat body during rest was 1.33 ± 0.47 µmol/gfw (n = 5), while that of glutamate was 1.75 ± 0.61 µmol/gfw (n = 5). There were no changes of the levels of these two amino acids observed after 30 min of flight and 60 min of rest thereafter.

Fat body contains about 155 µmol/gfw glycogen (Fig. 10B), which is much higher than in the flight muscles (65 µmol/gfw). Depletion of the glycogen stores took longer than in flight muscles. After 30 min, a concentration of around 60 µmol/gfw was

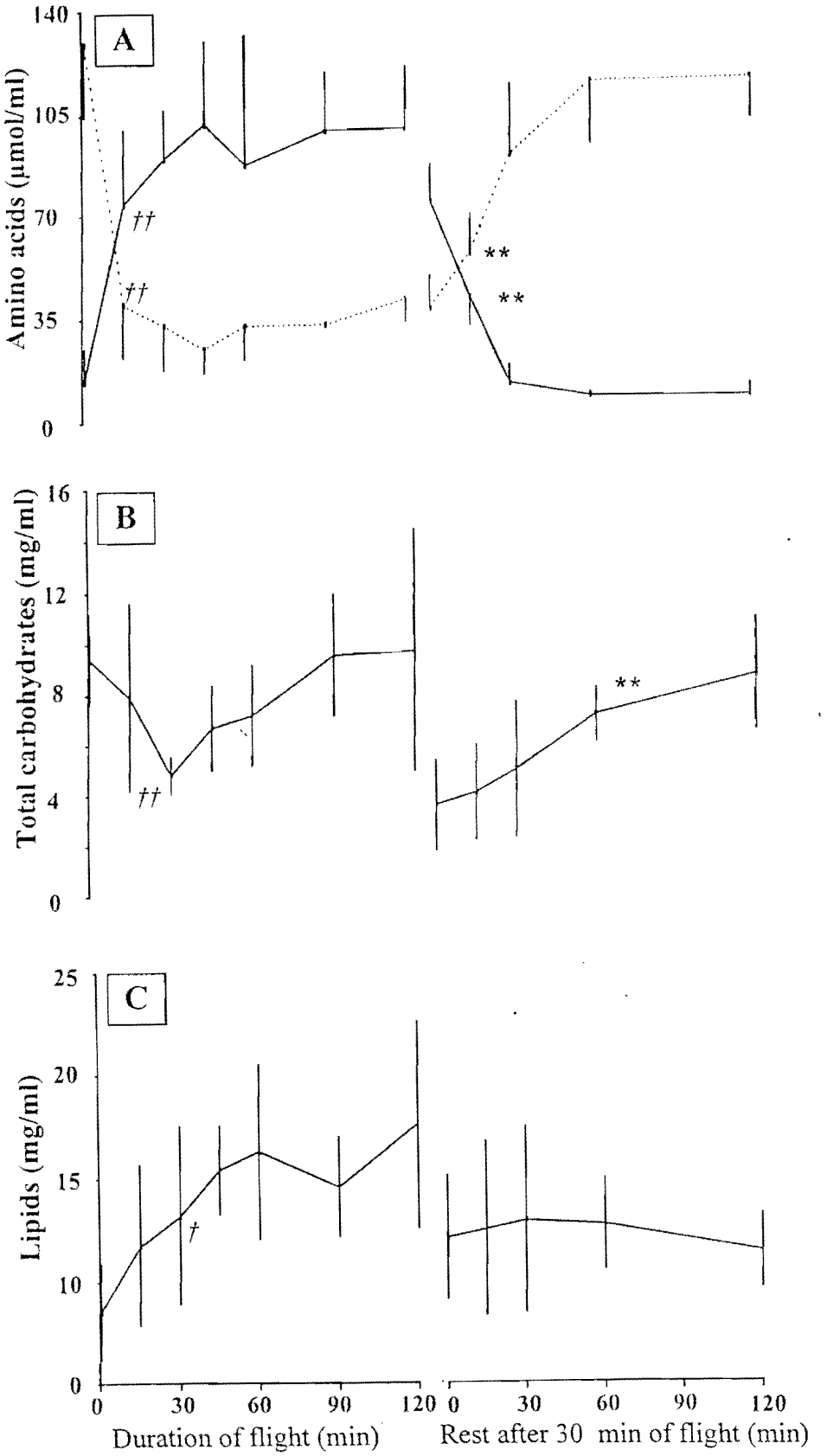

Figure 8. Metabolite concentrations in haemolymph of *P. sinuata* during different duration of flight without lift and subsequent rest after 30 min of flight: A. Proline (dashed line) and alanine (solid line) (n = 5 - 10); B. Total carbohydrates (n = 5 - 10); C. Lipids (n = 5 - 12). Values are given as means ± S.D. For significance levels see legend to Fig. 6. Reference values for beetles which flew were the resting values. For the resting period following flight, values were compared with those when flight was terminated.

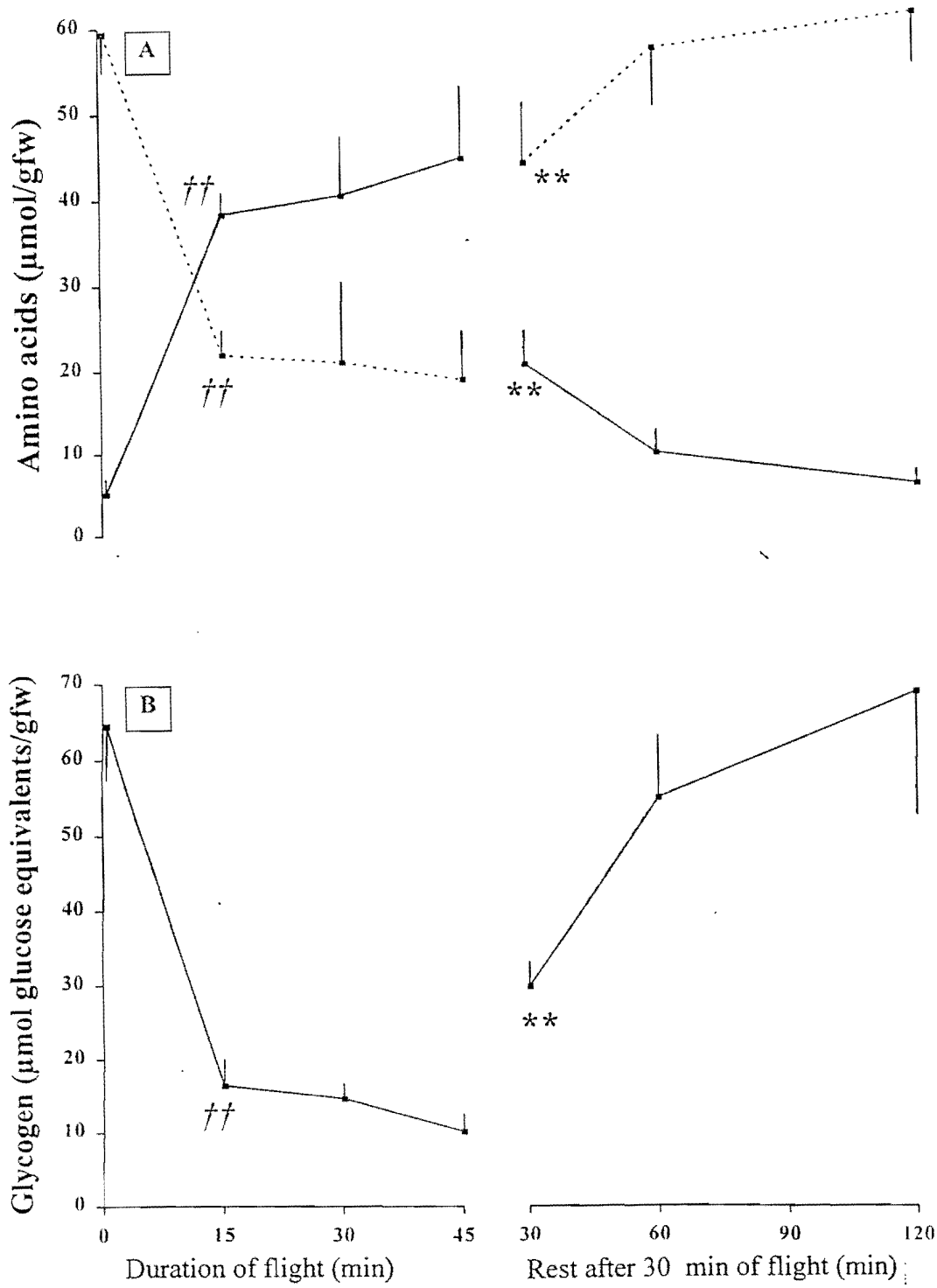

Figure 9. Metabolite concentrations in flight muscle tissue of *P. simuata* during different duration of roundabout flight and subsequent rest after 30 min of flight: A. Proline (dashed line) and alanine (solid line), B. Glycogen. Values are given as means ± S.D.; (n = 6). For significance levels see legend to Fig. 6. Reference values for beetles which flew were the resting values. For the resting period following flight, values were compared with those when flight was terminated.

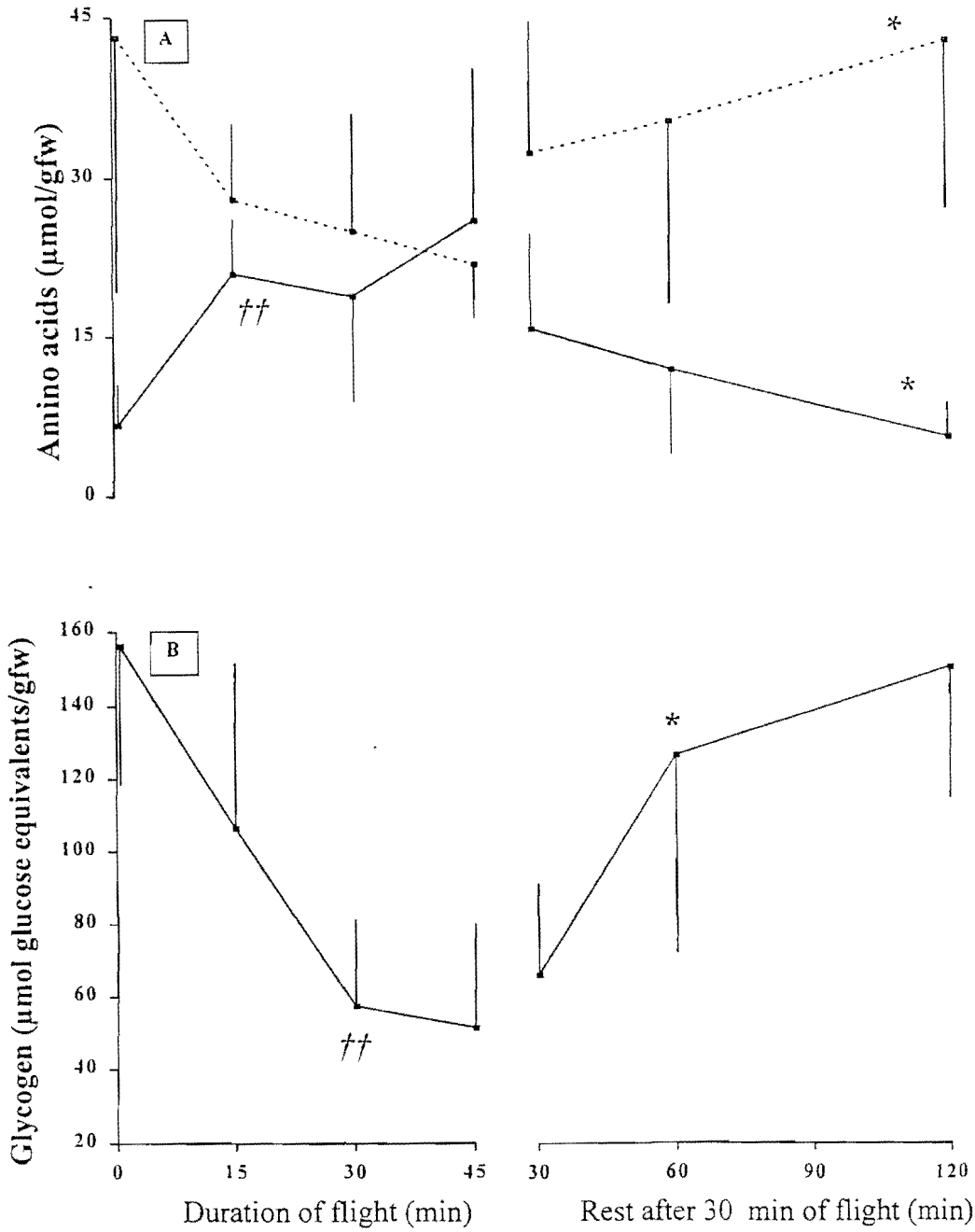

Figure 10. Metabolite concentrations in fat body tissue of *P. simulata* during different duration of roundabout flight and subsequent rest after 30 min of flight: A. Proline (dashed line) and alanine (solid line), B. Glycogen. Values are given as means ± S.D. (n = 5 - 7). For significance levels and reference values see legend to Figure 9.

reached, which remained unchanged even with longer flight times (Fig. 10B). Glycogen levels were restored to pre-flight values after 120 min of rest following 30 min of flight.

3.1.2.5. Flight velocity

Because it was not possible to measure flight performance directly during lift generating flight (only indirectly via oxygen consumption), the flight velocity during roundabout flight was recorded as a measure of performance (Fig. 11). Highest velocity was observed immediately after the commencement of flight at approximately 6 km/h. This dropped consistently until it stabilised at 3 km/h after about 30 min of flight.

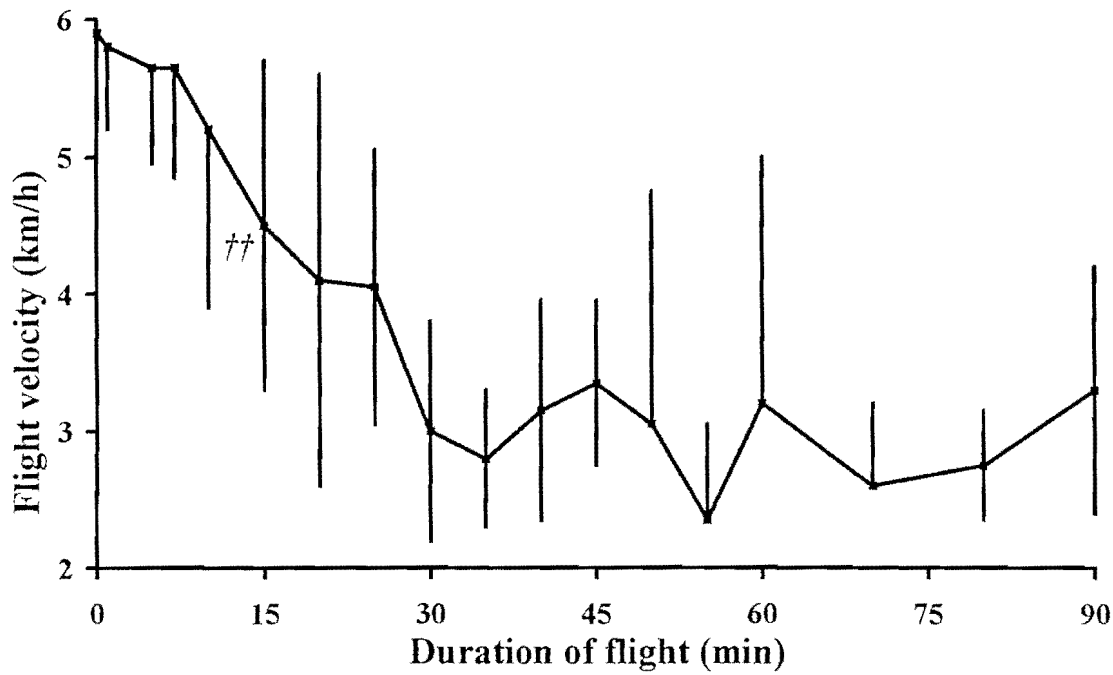

Figure 11. Flight velocity of *Pachnoda sinuata* during roundabout flight. Values are means ± S.D.; n = 3–10. For significance level see legend to Fig. 6. Reference value is the starting value.

3.1.3. Metabolic changes in different muscle types of *Pachnoda sinuata* during flight

To answer the question of whether the functional specialisation of the different muscles is accompanied by a biochemical/physiological specialisation, metabolic changes were measured separately in DLM, DVM and BM (see Fig. 2) after 30 s of lift generating flight and, to minimise lift, after different durations of roundabout flight (Fig. 12). Resting values of the investigated substrates showed no differences between the various muscles. Flight with lift of 30 s also did not reveal significant differences between muscles (Fig. 12A).

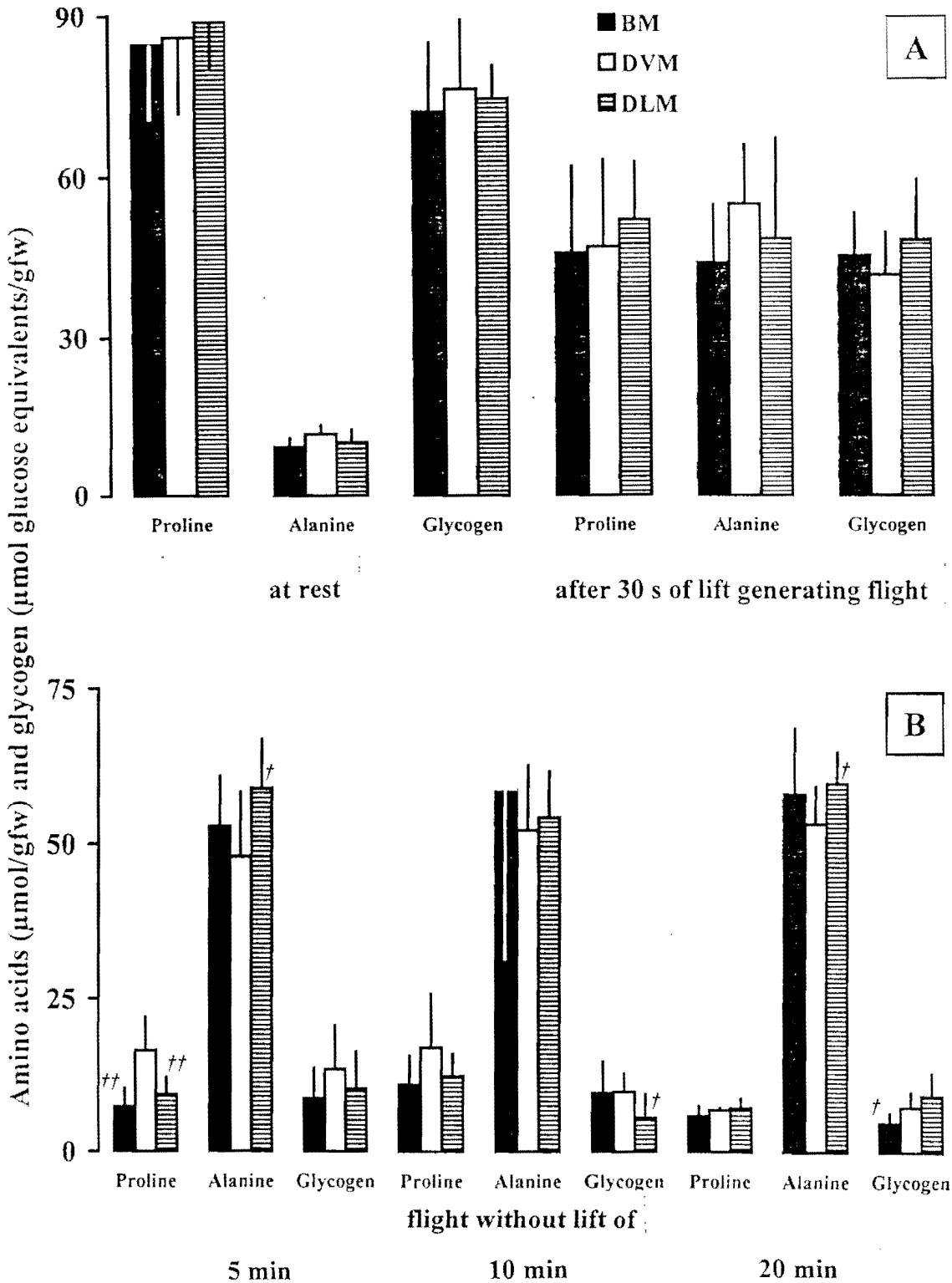

Figure 12. Metabolite concentrations in different flight muscles of *P. sinuata*: A. At rest and after lift generating flight (n = 5; each extract contained pooled material of two individuals), B. After several duration of roundabout flight (n = 7). Values are given as means ± S.D. Significance was calculated using Student's t-test. † $p < 0.05$, †† $p < 0.01$ compared with the value of DVM.

Flight on the flight mill, however, resulted in significant differences between the muscle types (Fig. 12B), with these differences largest in animals which flew for only 5 min. Proline and glycogen were highest in DVM in these animals and the amount of alanine generated from proline oxidation was smallest, in contrast to BM and DLM. The situation was similar after 10 and 20 min of flight, however, but differences were not as pronounced as those after a 5 min flight.

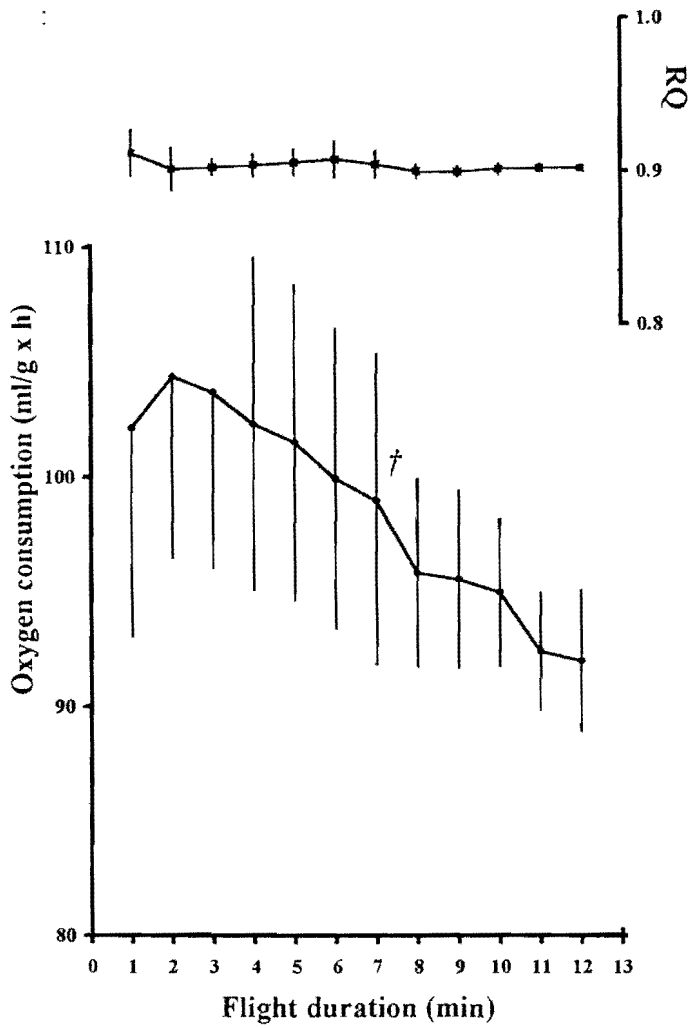

Figure 13. Time course of oxygen consumption and RQ of *P. sinuata* measured during lift generating flight. Data are given as means ± S.D., n = 3-6. (†) Significantly different compared with the value of 1 min of flight ($p < 0.05$).

3.1.4. Oxygen consumption and RQ

During lift generating flight, oxygen consumption (V_{O_2}) was highest shortly after the start with 104.4 ± 7.9 ml/g x h after 2 min, but dropped consistently with flight duration (Fig. 13). After 12 min of flight, the V_{O_2} value was 92.0 ± 3.1 ml/g x h. However, the RQ

remained at 0.9 throughout the duration of lift generating flight (Fig 13).

In Figure 14 the oxygen consumption during different activities of *P. sinuata* is compared. The resting value was 1.3 ± 0.4 ml/g x h, while during warm-up a maximum of 46.8 ± 7.0 ml/g x h was reached. V_{O_2} is in the same range with 43.8 ± 9.6 ml O_2/g x h during roundabout flight of 1 min, which is 42 % of the 104.4 ± 7.9 ml/g x h measured after 2 min of lift producing flight.

Resting RQ of *P. sinuata* was 0.89 ± 0.05 and similar values were measured during lift generating flight of 2 min (0.90 ± 0.01) as well as during roundabout flight of 1 min (0.89 ± 0.06). In contrast, a significantly lower value of 0.82 ± 0.05 was determined during pre-flight warm-up (Fig 15).

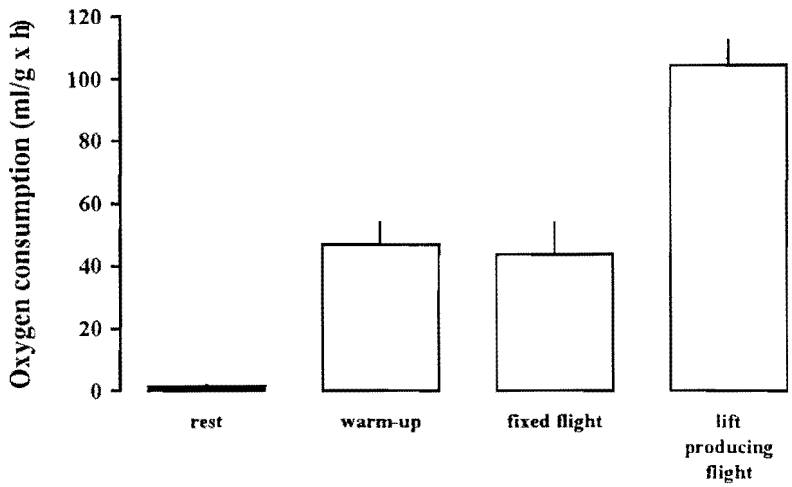

Figure 14. Comparison of oxygen consumption of *P. sinuata* during rest, warm-up, and flight with and without lift production. Data are given as means ± S.D., n = 6 - 8.

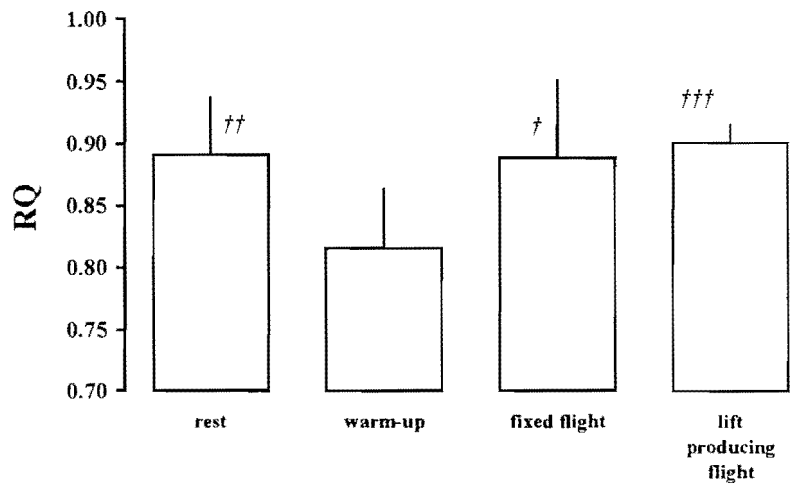

Figure 15. Comparison of RQ of *P. sinuata* during rest, warm-up, and flight with and without lift production. Data are given as means ± S.D., n = 6 - 10. Significance levels compared with warm-up value: † $p < 0.05$, †† $p < 0.01$, ††† $p < 0.001$ using Student's t-test.

3.1.5. Flight of the blister beetle, *Decapotoma lunata*

3.1.5.1. Concentrations of metabolites in the haemolymph

Haemolymph proline levels were highly variable (Fig. 16A). There was a decrease in proline concentration to 16.7 µmol/ml measured after 17 min of flight, which was significantly lower than the initial 34.8 µmol/ml. The high variability of the values during rest suggests that beetles do not recover simultaneously. Although the level after 2 h of rest was higher than that when beetles had stopped after 10 min of flight, no significant differences occurred. The alanine levels were significantly increased from the initial 1.8 µmol/ml to 6.6 µmol/ml after 10 min of flight, when the concentrations plateaued (Fig. 16A). Alanine concentration returned to the initial pre-flight levels 2 h after a 10 min flight.

Total carbohydrate levels in the haemolymph also showed a high degree of variability (Fig. 16B). A significant reduction was recorded after a 10 min flight. The high scatter of values during the rest period after flight experiments, does not allow any conclusions to be drawn. It would appear that recovery is a protracted process, because only the carbohydrate value measured after 2 h differed significantly from the 10 min flight value.

The concentration of total lipids in the haemolymph did not change significantly during flight; 12.7 ± 2.2 mg/ml (mean ± S.D.; n = 7) was measured in resting beetles, 13.7 ± 4.6 mg/ml (n = 15) after 10 min of flight and 14.4 ± 4.0 mg/ml (n = 8) 30 min after a flight of 10 min (not depicted).

3.1.5.2. Concentrations of metabolites in the thorax

Proline concentrations decreased continuously during flight, apparently at a higher rate at the onset of flight (Fig. 17A). A significant drop from the initial 49.3 µmol/gfw to 35.5 µmol/gfw was clearly evident after 2 min of flight. Restoration of pre-flight levels was slow and took 2 h. Concentrations of alanine followed an opposite pattern to that of proline, resulting in a constant sum of 40-50 µmol/gfw for the two amino acids throughout the experiments, with the exception of a lower value for 17 min flight (30.5 µmol/gfw; Fig. 17A).

During the first 2 min of flight, glycogen levels remained unchanged, but decreased significantly over the next 8 min, from an initial 27 µmol glucose equivalents/gfw to 7.5 µmol/gfw at which level it stayed until 17 min of flight (Fig. 17B). During a 1 h rest period following a 10 min flight, glycogen levels returned to pre-flight values.

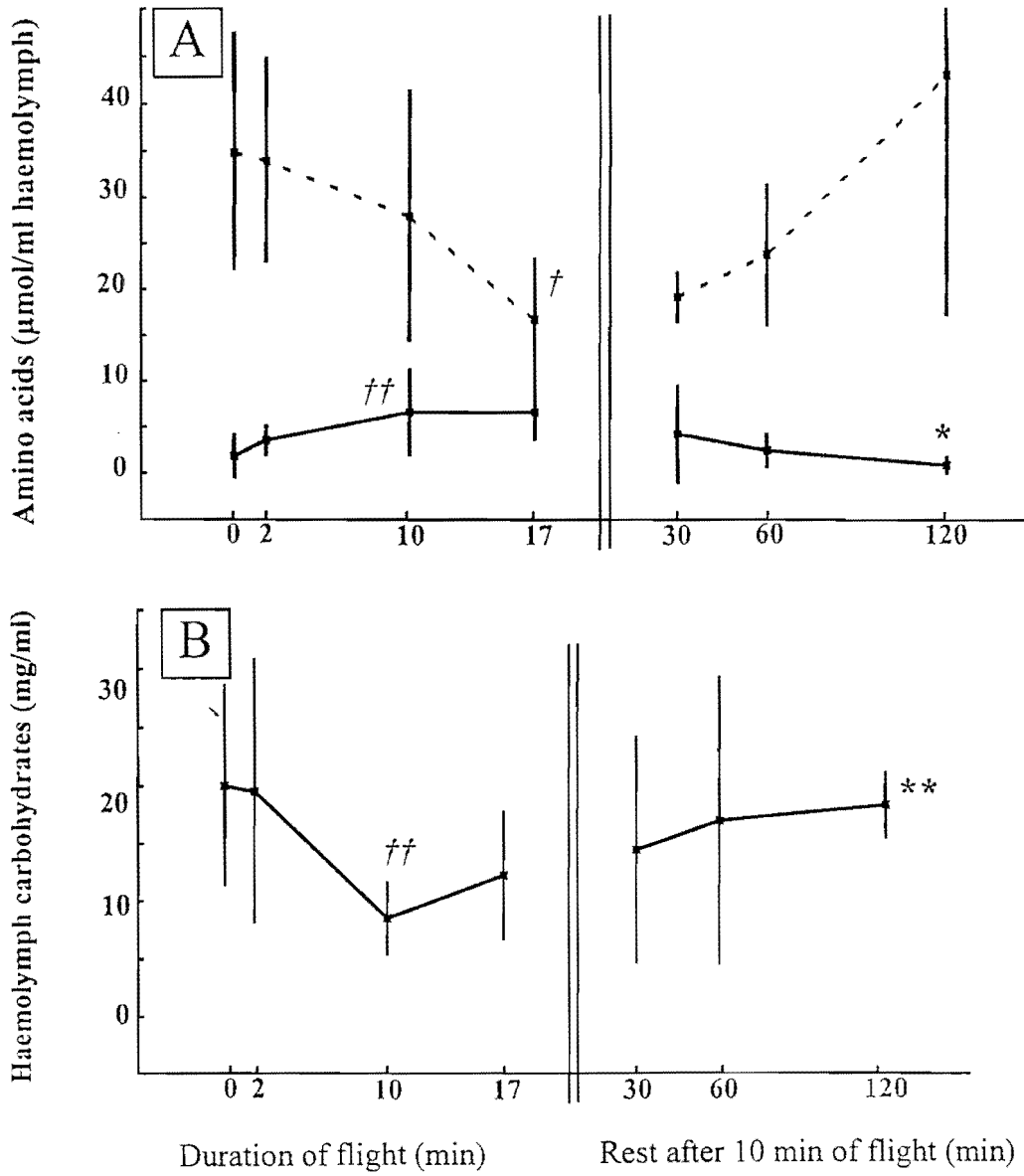

Figure 16. Concentration of: A. proline (dashed lines) and alanine (solid lines) and B. total carbohydrates in the haemolymph of *D. lunata* during various times of flight and a subsequent rest period after 10 min of flight. Values are given as means ± S.D.; n = 4-23. For significance levels see legend to Fig. 6. The reference values for beetles which flew were the resting levels.

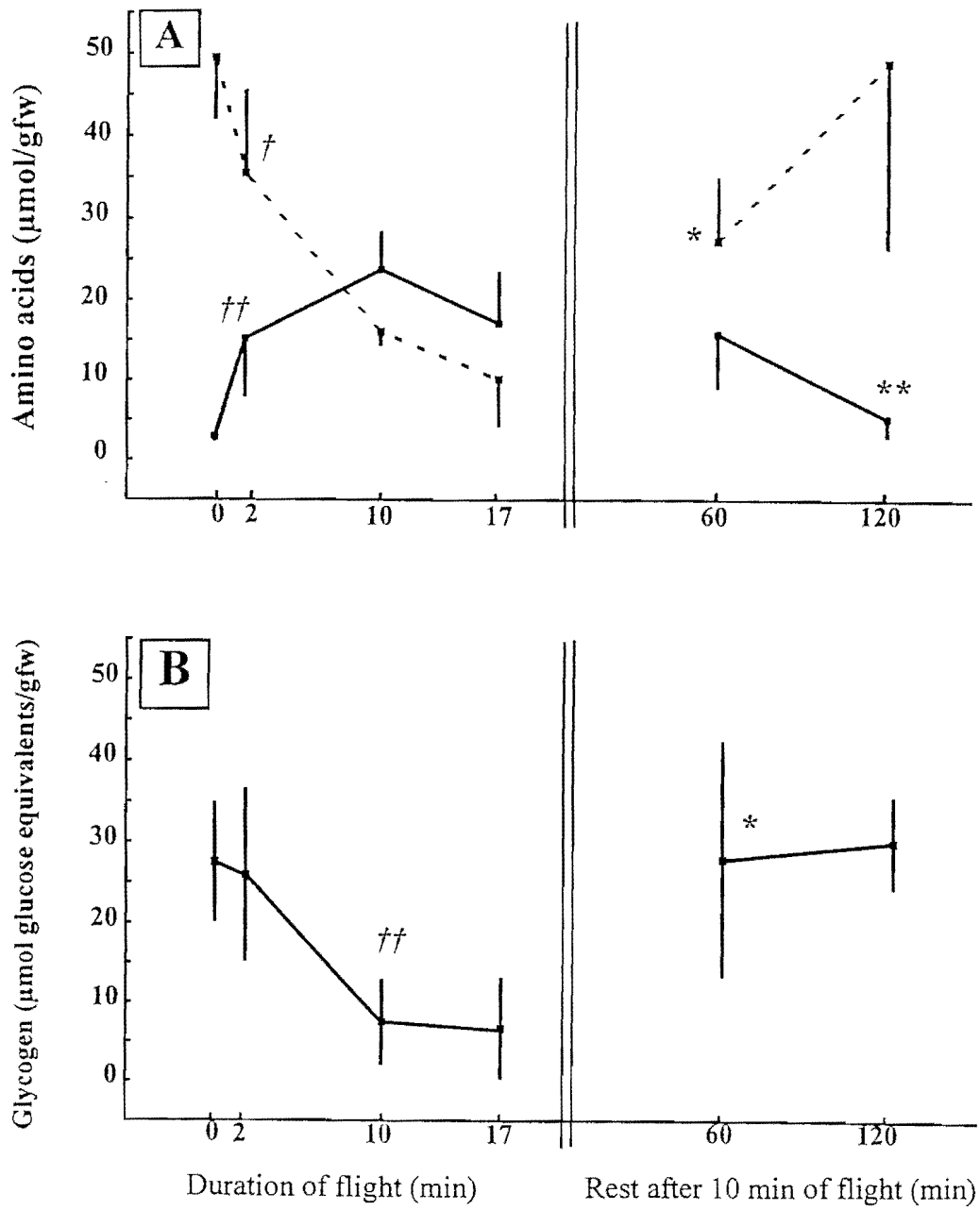

Figure 17. Concentrations of: A. proline (dashed lines) and alanine (solid lines) and B. glycogen in the thorax of *D. lunata* during various times of flight and during a rest period following 10 min of flight. Values are given as means ± S.D.; n = 4-7. For significance levels see legend to Fig. 6. Values of flying beetles were compared with resting values.

3.1.6. Flight of the Protea beetle, *Trichostetha fascicularis*

3.1.6.1. Metabolic changes in the haemolymph

Resting levels of haemolymph lipids were low (6.1 ± 1.1 mg/ml) and did not change during lift generating flight of 1 min (5.9 ± 1.1 mg/ml) or subsequent rest of 60 min after flight (5.9 ± 0.3 mg/ml; Fig. 18A).

The haemolymph carbohydrate levels increased from 12.5 to 18.2 mg/ml after 1 min of flight (Fig. 18A). This seems to resemble the pattern in *P. sinuata* (see section 3.1.1.), where carbohydrates increased for a short period after the onset of flight. The value for rest after flight was lower than the resting value (8.5 mg/ml).

Similar to *P. sinuata*, proline levels in the haemolymph dropped dramatically from nearly 100 to about 60 µmol/ml during flight, accompanied by a sharp increase of haemolymph alanine from about 2 to 40 µmol/ml. After 60 min of rest thereafter, pre-flight levels were reached.

The behavioural pattern during the pre-flight warm-up phase in *T. fascicularis* was similar to that described in section 2.3.2. for *P. sinuata*, but was not investigated in this beetle. Therefore, the difference between the resting values and the respective flight values included possible metabolite changes during this process.

3.1.6.2. Metabolic changes in the flight muscles

During 1 min of flight, glycogen concentration fell from more than 60 to about 40 µmol glucose equivalents/gfw, but increased to 58 µmol glucose equivalents/gfw during subsequent rest of 60 min (Fig. 18B).

Proline levels of 65 µmol/gfw were depleted to about 40 µmol/gfw after 1 min of flight. Thereafter, the resting level was reached during 60 min of rest.

Concomitantly, alanine levels increased from 6 to 36 µmol/gfw during flight, dropping to 4 µmol/gfw during 60 min of rest thereafter (Fig. 18B).

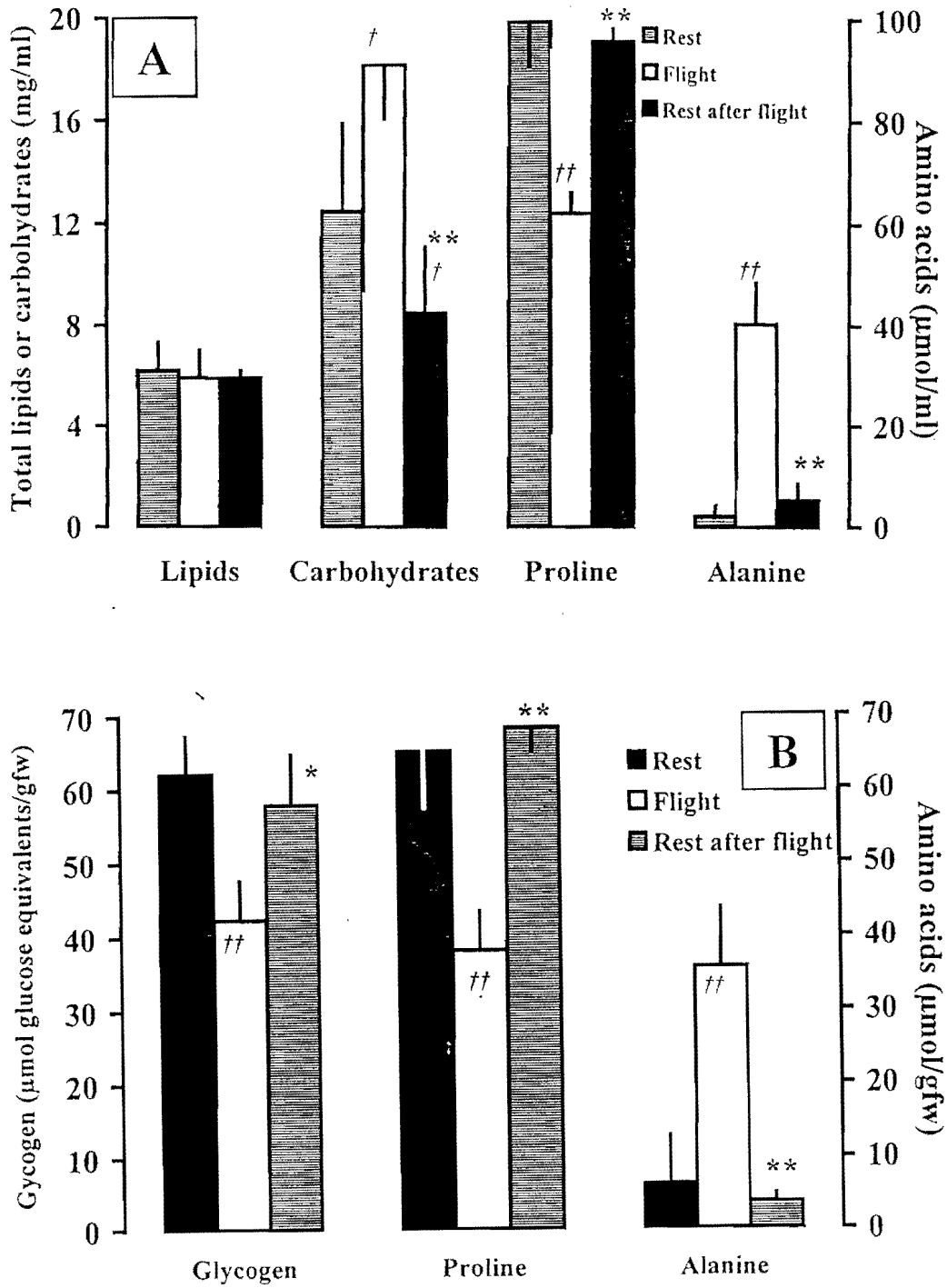

Figure 18. Metabolite concentrations in (A) haemolymph and (B) flight muscles of *Trichostetha fascicularis* during rest, after 1 min of flight, and after subsequent rest of 60 min. Values are given as means ± S.D.; n = 5 (A) and n = 6 (B). Significance levels of differences compared with the resting groups are † $p < 0.05$ and †† $p < 0.001$. Significance levels of difference compared with those beetles which flew: * $p < 0.05$, ** $p < 0.001$.

3.1.7. Flight experiments with carpenter bees, *Xylocopa capitata*

Many hymenopterans are known to be exclusive carbohydrate users (Sacktor, 1975). Therefore, carpenter bees were included in the investigations for comparative reasons.

Carpenter bees, *Xylocopa capitata*, had negligible amounts of lipids in their haemolymph. The resting value was 0.7 ± 0.6 mg/ml (n = 7) which dropped to 0.1 ± 0.3 mg/ml after 10 min of lift generating flight, reflecting a significant difference of 0.6 ± 0.4 mg/ml ($p < 0.02$). After a rest period of 2 h thereafter, lipid concentration was 0.2 ± 0.5 mg/ml. The bees had a very high level of total haemolymph carbohydrates, 36.7 ± 15.7 mg/ml at rest (n = 6) dropping to 15.7 ± 8.6 mg/ml during 10 min of flight. The difference of 19.3 ± 16.4 was significant ($p < 0.05$). When the animals rested for 2 h after flight, 14.6 ± 7.1 mg/ml were measured, which suggested that the bees were not able to restore pre-flight levels without feeding. The proline concentration in carpenter bees at rest was 7.3 ± 6.2 µmol/ml (n = 5). After 1 min of flight no proline or alanine could be detected in the haemolymph of the bees. However, after 2 h of subsequent rest, 4.9 ± 3.5 µmol/ml of proline were measured in the haemolymph.

During flight of 2 min, the carpenter bees consumed 75.0 ± 5.3 ml O_2/g x h (n = 4). Their RQ was found to be 1.04 ± 0.07 (n = 4), which confirms the exclusive oxidation of carbohydrates.

3.1.8. Resting haemolymph metabolite concentrations in several insects

The following haemolymph proline concentrations were measured during rest of a number of insects: *L. migratoria*, 9.2 ± 1.5 µmol/ml (3); *P. americana*, 13.6 ± 5.8 µmol/ml (3); *D. cincta*, 106.3 ± 4.8 µmol/ml (5); *M. melolontha*, 75.2 ± 10.9 µmol/ml (6); *Lepithrix* sp. 37.1 ± 3.1 µmol/ml (4); *C. innocua*, 34.9 ± 10.1 µmol/ml (6); *Cicindela* sp., 25.7 ± 4.7 µmol/ml (5); *T. molitor*, 10.6 ± 0.1 µmol/ml (6); *Julodes cirrosa*, 13.0 ± 3.6 µmol/ml (4); *P. capensis*, 0 (4) and *H. eson*, 3.4 ± 0.4 µmol/ml (4). Values are means ± S.D. with n in brackets.

The resting lipid concentrations were: *L. migratoria*, 9.9 ± 1.9 µmol/ml (4); *P. americana*, 15.7 ± 0.9 mg/ml (7); *M. melolontha*, 6.2 ± 1.1 mg/ml (5), *C. innocua*, 7.1 ± 2.5 mg/ml (4); *D. cincta*, 6.7 ± 2.1 mg/ml (3); *Cicindela* sp., 5.4 ± 2.9 mg/ml (5); *Lepithrix* sp., 4.8 ± 2.5 mg/ml (6).

Carbohydrate levels at rest were: *L. migratoria*, 26.8 ± 4.2 mg/ml (4); *Lepithrix* sp., 9.2 ± 3.5 mg/ml (6); *M. melolontha*, 8.2 ± 4.3 mg/ml (5); *C. innocua*, 13.1 ± 3.7 mg/ml (6) and *D. cincta*, 106.3 ± 2.8 mg/ml (5).

The above data are used for comparison in Table 24 in Discussion.

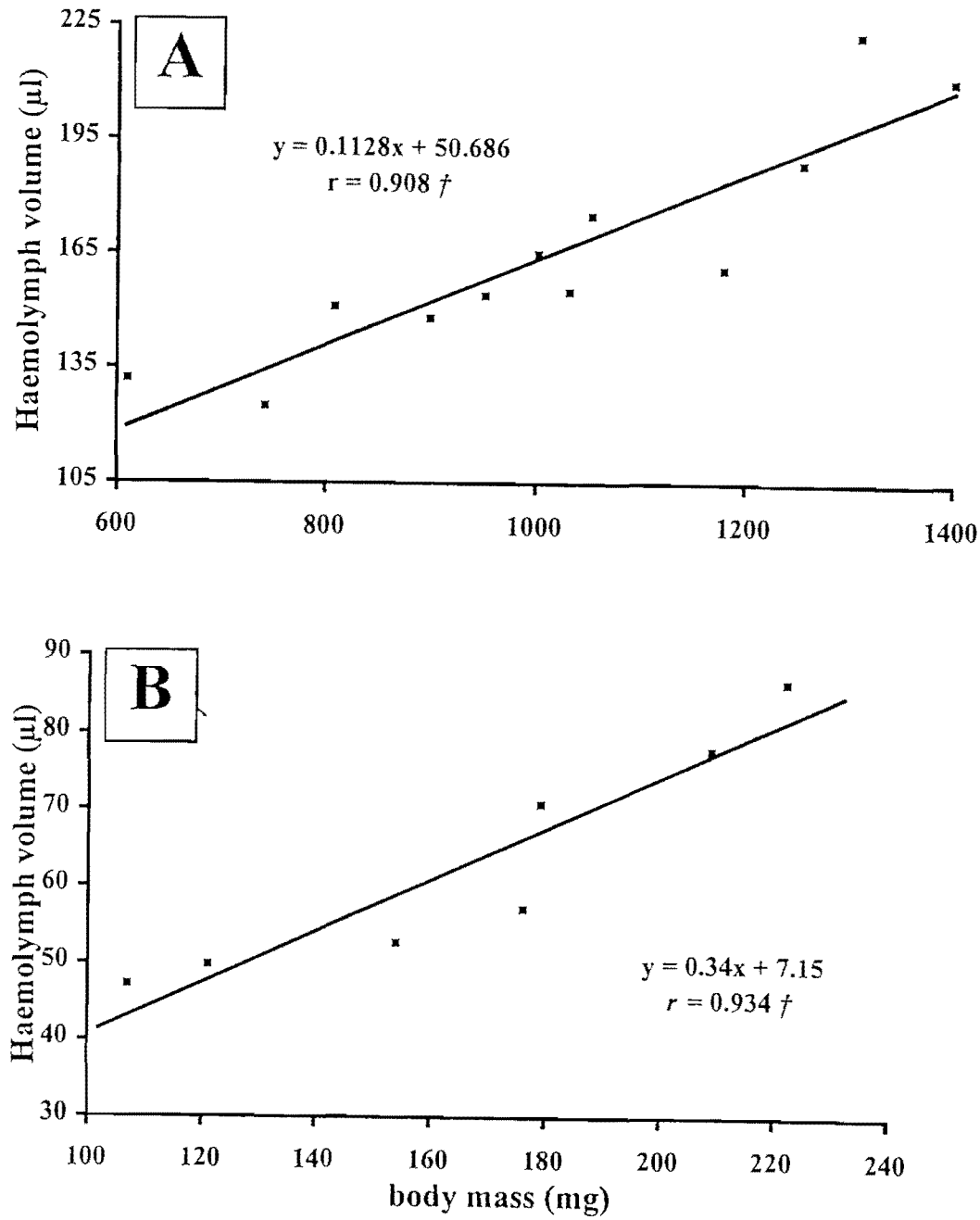

Figure 19. Relationship between body weight and haemolymph volume in: A. *Pachnoda sinuata* and B. *Decapotoma lunata*. Significance level of the correlation: † $p < 0.001$.

3.1.9. Haemolymph volume and tissue weight

3.1.9.1. Pachnoda sinuata

To estimate the participation of proline and carbohydrates and the role of different body parts to the supply of energy during flight, oxygen consumption was calculated, for which it was necessary to know the haemolymph volume. A significant linear relationship between body weight (w) and haemolymph volume (V) was shown for 12 beetles of unknown age with a body weight of 1020 ± 237 mg (ranging from 610 to 1400 mg body weight) (Fig 19A). It followed the equation: V (µl) = 0.113 w (mg) + 50.69, $p < 0.001$. The mean haemolymph volume for those beetles was 165.8 ± 29.5 µl. Flight muscles were found to represent 8.2 ± 1.9 % (n = 5) of total body weight, and the fat body tissue 4.1 ± 3.8 % (n = 6). Thus, a beetle with the average fresh weight of 1020 mg contained 83.6 mg flight muscles, 41.8 mg fat body tissue and 166 µl haemolymph.

3.1.9.2. Decapotoma lunata

When the haemolymph volume was determined, a significant linear relationship between the beetle's total weight and haemolymph volume (V) was established, following the equation: V (µl) = 0.34 w (mg) + 7.15; $r = 0.934$; $p < 0.001$ (Fig. 19B). Thus, a mean haemolymph volume of 63.2 ± 15.3 µl (± S.D., n = 7) was established for beetles with an average mass of 166.9 ± 42.6 mg.

3.2. Pre-flight warm-up of *Pachnoda sinuata*

3.2.1. Body temperature and flight performance

Figure 20 depicts the relationship of thoracic temperature (T_{th}) and wing beat frequency (WBF) of beetles which were flown at ambient temperatures (T_a) of 25 °C and 31 °C. The highest WBF (at 25°C) were recorded immediately after the onset of flight; later WBF dropped with decreasing thoracic temperature. The decrease of WBF and T_{th} correlated at an ambient temperature (T_a) = 25°C (Fig. 20). During flight at T_a = 31°C, WBF and T_{th} stayed relatively stable. Because smaller beetles lose relatively more heat than larger ones (Bartholomew and Heinrich, 1978), data from two representative beetles (similar body size) were chosen to show the difference in WBF and T_{th} at different T_a (Fig. 21). At a T_a of 25 °C, the beetle would warm up to 37 °C and started flying voluntarily. During flight, T_{th} did not increase further but began decreasing in the first min of flight (Fig. 21). Although a T_{th} of

31°C is sufficiently high for take-off, the beetle would still warm up further to about 36 °C at T_a = 31 °C (Fig. 21). The optimal flight temperature for *P. sinuata* therefore should be between the two T_a's investigated, when heat loss equals heat production.

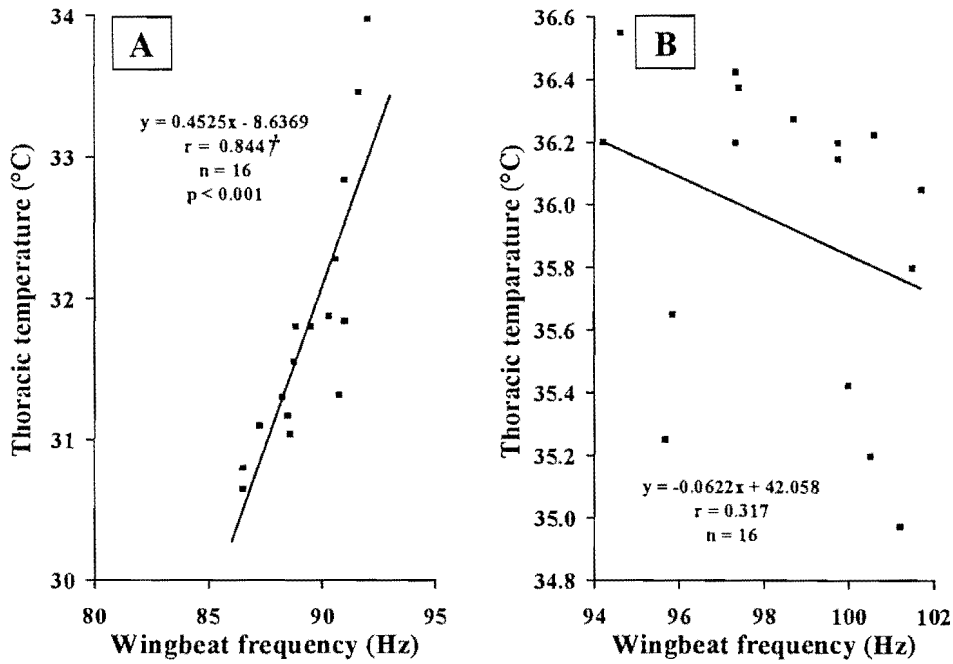

Figure 20. Relationship between thoracic temperature and wing beat frequency of *P. sinuata*, during fixed flight at A: T_a = 25 °C and B: 31 °C. Data points are means of 3-6 individuals. Significance level of correlation: † $p < 0.001$.

As shown in Figure 22, T_{th} of *P. sinuata* were measured at different phases of activity in the field and in the laboratory. Beetles resting in the shade (underneath leaves) had a T_{th} of 24.6 ± 0.9 °C, while T_{th} of those resting in bright sunlight was 31.1 ± 1.6 °C. The latter beetles were able to take off for flight without any visible warm-up or starting preparations as described in section 2.3.2., which are also known from the cockchafer, *Melolontha melolontha* (Schneider, 1980). In some beetles the T_{th} was measured as 33.8 ± 1.9 °C after endothermic warm-up in the laboratory (T_a = 25 °C) and exposure to sunlight from a window (thus, similar conditions as in the field). Beetles which walked slowly (not exposed to direct sunlight) had a lower T_{th} of 27.4 ± 0.8 °C. Some of the beetles which had been resting on leaves or flowers (i.e. not exposed to direct sunlight) were forced to fly, without warming up, by dropping them from a 1 m height (T_a > 25°C). When they opened their wings and tried to fly under these conditions, T_{th} was measured and found to be 27.9 ± 1.5 °C. The T_{th} was also measured in some beetles which had just returned to the plant after an unknown duration of flight. These individuals were either caught in the air before landing or were picked from the plant just after landing. T_{th} in these beetles was 32.1 ± 1.1°C. The results indicate that T_{th}

below *ca.* 28°C are not sufficient for flight in *P. simuata*.

To determine the duration of warm-up (from beginning of T_{th} increase until take-off), T_{th} was measured at T_a of 25 °C. The beetles elevated their T_{th} from 25.9 ± 0.2 °C to 34.5 ± 2.0 °C in 83 ± 23 s (n=8).

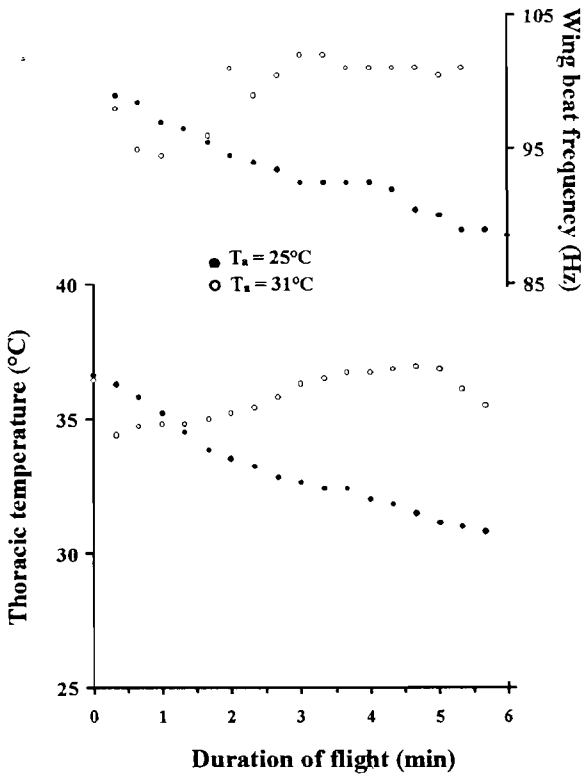

Figure 21. Wing beat frequency and body temperature during fixed flight of *P. simuata* at two different ambient temperatures. The figure shows a representative example (body weight of about 1000 mg) for each ambient temperature.

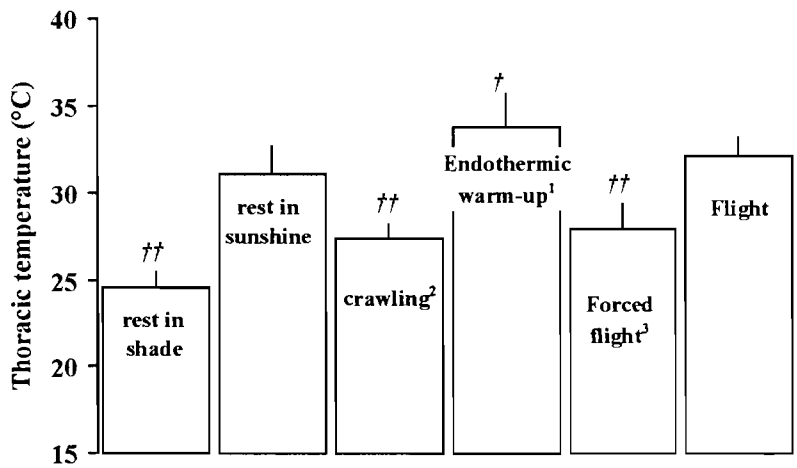

Figure 22. Thoracic temperatures of *P. simuata* during different activities. Values are means ± S.D; T_a = 24.5 - 25.2 °C. [1]Measured in the laboratory at T_a = 25 °C close to a window. [2]Not exposed to direct sunlight. [3]Beetles were resting in the shade when captured. Significance of the difference compared with beetles arriving from flight: † $p < 0.05$, †† $p < 0.001$.

3.2.2. Metabolic fuels during warm-up

As can be seen from Figure 23, the glycogen concentrations in the flight muscles were not affected by warm-up, and remained at 58.8 ± 9.3 µmol glucose equivalents/gfw compared with 60.8 ± 9.1 µmol/gfw at rest. However, glycogen levels fell to 45.3 ± 8.1 µmol glucose equivalents/gfw during the following 10 s of lift generating flight.

The proline concentration in the flight muscles dropped significantly during warm-up from 53.5 ± 12.1 to 34.7 ± 9.2 µmol/gfw, decreasing further to 27.1 ± 4.9 µmol/gfw during 10 s of flight (Fig. 23). In contrast, the alanine concentration in the flight muscle increased from 3.1 ± 1.8 to 15.4 ± 5.6 µmol/gfw during warm-up and to 18.0 ± 2.6 µmol/gfw during 10 s of flight (Fig. 23).

To substantiate these results, haemolymph samples were taken at rest, after warm-up and after 10 s of subsequent flight from individual beetles to determine the proline, alanine, carbohydrate and lipid concentrations. Although the variability of the resting values was high, most of the 10 beetles used for the results in Figure 24A clearly exhibited a drop in the proline concentration during warm-up and a further drop during a subsequent 10 s of flight. All individuals showed a sharp increase of alanine during warm-up which continued during 10 s flight (Fig. 24B).

Concentrations of total carbohydrates in the haemolymph of individual beetles did not change significantly during warm-up and subsequent 10 s of flight. The values for 24 beetles were 13.0 ± 5.4, 13.5 ± 5.6 and 14.3 ± 5.3 mg/ml, respectively.

Haemolymph lipids also did not change, with concentrations remaining at around 10 mg/ml.

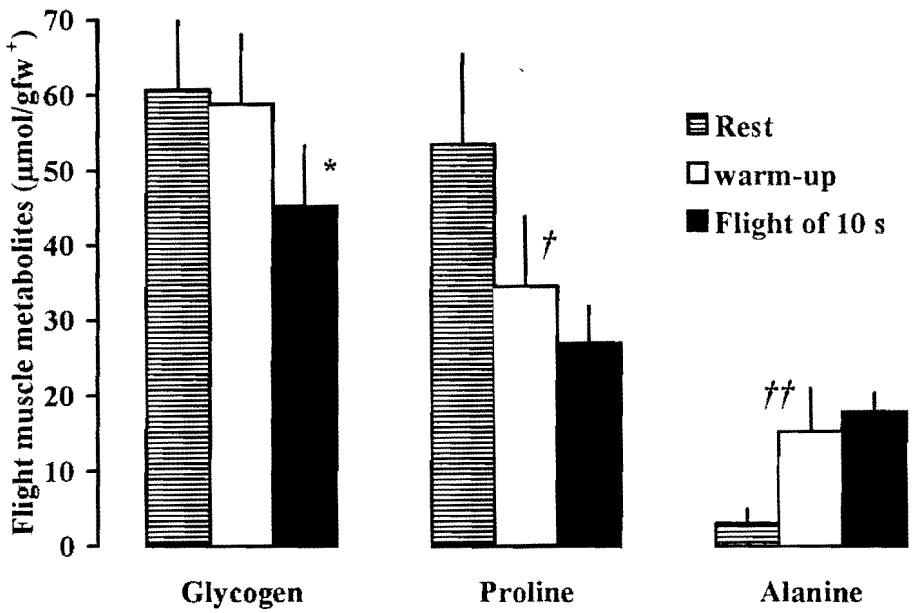

Figure 23. Concentrations of glycogen, proline and alanine in flight muscle tissue of *P. sinuata* at rest, after warm-up and after 10 s of flight following warm-up. Data are given as means ± S.D., n = 6. Significance levels of warm-up value compared with resting value: † $p < 0.02$, †† $p < 0.001$; flight value compared with warm-up value: * $p < 0.05$ using Student's t-test. +Glycogen is expressed as µmol glucose equivalents/gfw).

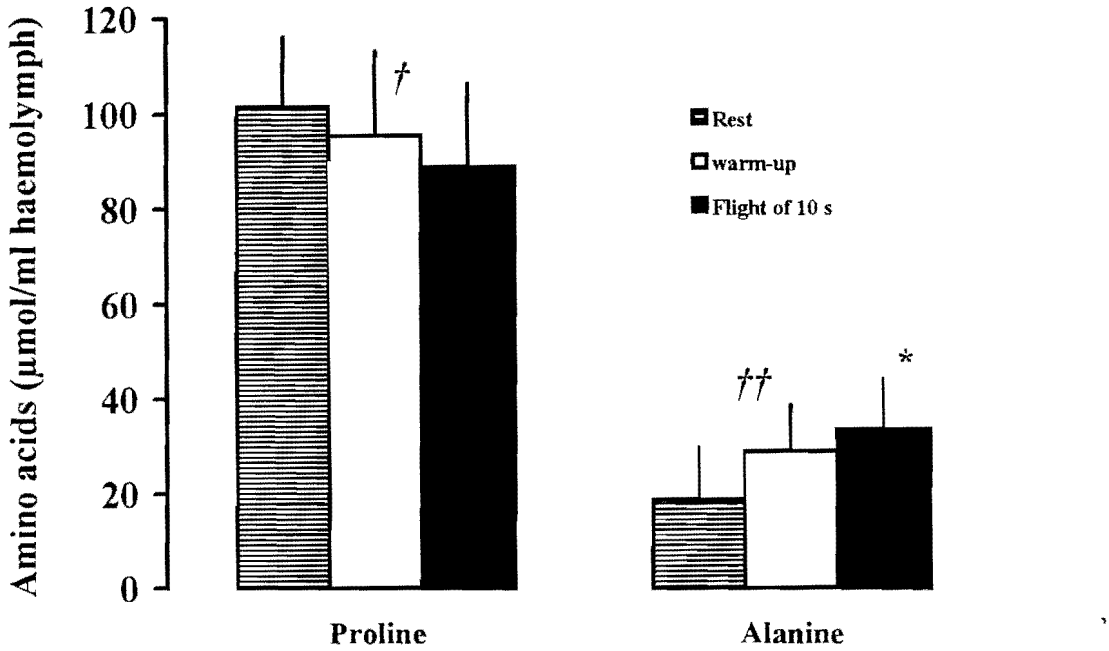

Figure 24. Concentration of proline and alanine in the haemolymph of individual *P. sinuata* during rest, warm-up and subsequent lift generating flight of 10 s. Data are given as means ± S.D., n = 10. Significance levels of warm-up value compared with resting value: † $p < 0.05$, †† $p < 0.001$; flight value compared with warm-up value: * $p < 0.005$ using paired t-test.

3.3. Starvation experiments

3.3.1. Metabolites in the haemolymph

The concentration of proline in the haemolymph remained relatively stable during the whole period of starvation when compared with the other metabolites (Fig. 25A). The proline concentration rose from an initial value of 94.6 ± 6.0 µmol/ml to 106.6 ± 9.9 µmol/ml after 6 days of starvation. Later the concentration dropped and reached 72.4 ± 13.2 µmol/ml after 28 days of starvation. At day 31 of starvation, animals were re-fed. Proline concentration did not change, and 24 h after feeding it was 71.8 ± 10.2 µmol/ml.

The alanine concentration decreased during starvation and after 14 days almost no alanine could be measured (1.1 ± 1.3 µmol/ml). Two hours after re-feeding following 31 days of starvation, the alanine concentration rose to almost normal levels reaching 9.9 ± 5.7 µmol/ml 24 h after re-feeding.

The most dramatic change during starvation was found in the carbohydrate levels of the haemolymph (Fig. 25B), with levels dropping from 10.2 ± 4.1 mg/ml to 5.2 ± 1.5 mg/ml after only 4 days of starvation. This trend continued until about day 14, when the concentration stabilised at a low level of about 1-2 mg/ml. At day 31 of starvation, levels had reached 0.3 ± 1.2 mg/ml. After re-feeding, concentrations rose steeply to 7.8 ± 2.4 mg/ml and further to 13.0 ± 6.9 mg/ml 24 h after re-feeding. This value was not different from the pre-starvation value.

No significant changes of the concentration of haemolymph lipids occurred during the starvation period of up to 31 days. When the beetles were re-fed after 31 days of starvation lipid concentrations also did not change.

3.3.2. Metabolites in the flight muscles

Concentrations of proline, alanine and glycogen were measured in fed animals and in those which had starved for 15 and 30 days (Fig. 26). Proline concentration was 62.1 ± 5.8 µmol/gfw in fed animals and 56.6 ± 18.7 and 50.9 ± 10.4 in those which had starved for 15 and 30 days, respectively. Alanine concentration was 4.4 ± 2.1 , 5.3 ± 3.2 and 2.2 ± 0.8

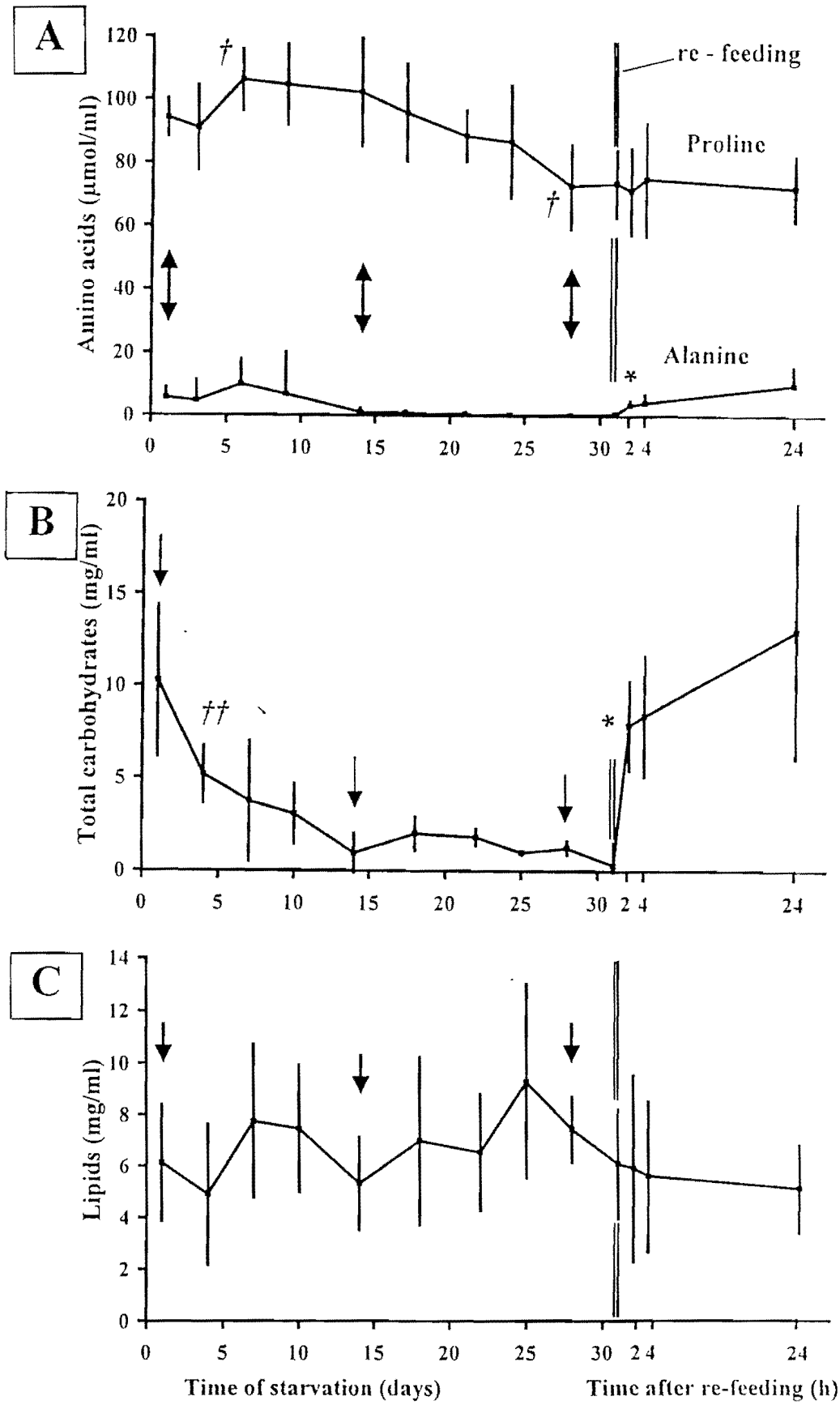

Figure 25. Metabolic changes in the haemolymph of *P. sinuata* during starvation. A: Proline and alanine; B: Carbohydrates; C: Lipids. Values are means ± S.D. of 4 - 6 animals. Arrows indicate when bioassays were performed (see Tables 4 and 5). Significance levels: † $p < 0.05$ and †† $p < 0.001$ compared with fed beetles; * $p < 0.001$ compared with beetles before re-feeding.

µmol/gfw in the respective period.

Dramatic changes occurred in the glycogen stores (Fig. 26). The initial concentration was 59.2 ± 9.7 µmol glucose equivalents/gfw, which decreased to 8.8 ± 4.3 and 5.3 ± 1.9 µmol glucose equivalents/gfw in those animals which had starved for 15 and 30 days, respectively.

3.3.3. Metabolites in fat body

Concentrations of proline, alanine and glycogen were measured in fed animals and in those which had starved for 15 and 30 days (Fig. 26). Proline levels did not change significantly during the starvation period and large individual differences occurred. Alanine concentration did not change significantly during 15 or 30 days of starvation.

Glycogen content was high in fed animals (152.3 ± 63.8 µmol glucose equivalents/gfw) and very low in animals which had starved for 15 and 30 days (31.1 ± 22.7 and 8.1 ± 12.6 µmol glucose equivalents/gfw, respectively).

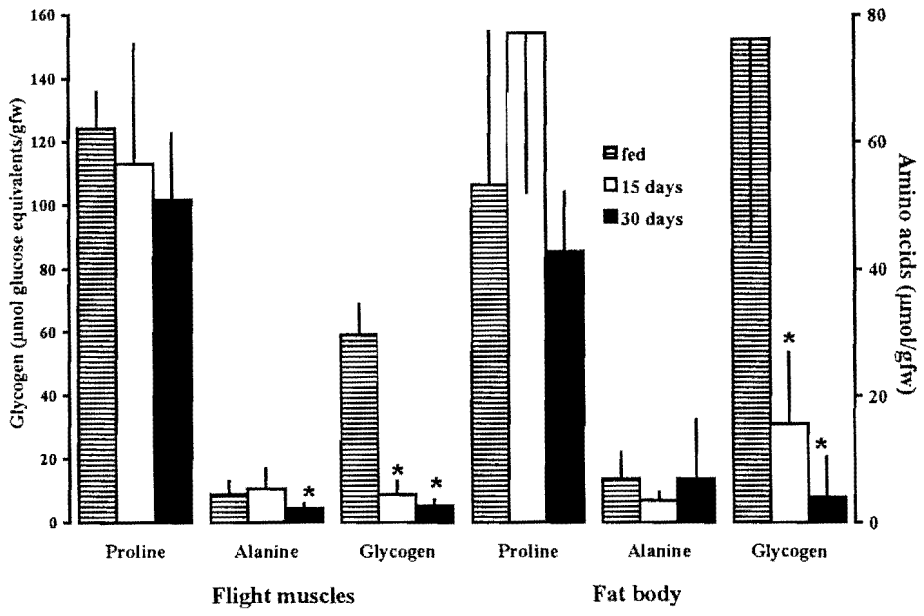

Figure 26. Metabolite concentration in flight muscles and fat bodies of *P. sinuata* after different periods of starvation. Values are given as means ± S.D., n = 5. Significance of difference compared with the value of fed animals: * $p < 0.005$.

3.3.4. Bioassays during starvation

The AKH peptide of *P. sinuata*, Mem-CC, was shown to cause an increase of haemolymph carbohydrate concentration (Lopata and Gäde, 1994) and of haemolymph proline concentration (see section 3.7.). It was thought to be a useful tool to investigate whether the animals are still able to release energy metabolites from the fat body and other stores during the starvation period. Therefore, bioassays in which 50 pmol Mem-CC was injected (to ensure maximum response; see section 3.7.) were conducted at different days of the starvation period (Tables 3 and 4).

It could be shown that the injected Mem-CC caused an increase in haemolymph proline concentration after starvation, but the increase was only 9.6 ± 10.4 µmol/ml after 14 days of starvation and after 28 days, no change occurred, while it was 18.4 ± 12.8 µmol/ml at day 1 of starvation. Concomitantly, the decrease of haemolymph alanine concentration was 5.0 ± 2.7 µmol/ml on day 1 of starvation, but only 1.4 ± 1.4 in animals which had starved for 14 days. After 28 days no changes were found. The pre-injection values of alanine for the two bioassays conducted after 14 and 28 days of starvation was lower than those obtained after one day of starvation (see Fig. 25 and Table 3). At day one of starvation, the increase of haemolymph carbohydrates was 6.5 ± 2.1 mg/ml, while there was no significant increase after 14 and 28 days, respectively (Table 4). No influence of Mem-CC on the haemolymph lipid concentration was found after different starvation periods (Table 4).

Table 3. Bioassays during starvation experiment. Injection of 50 pmol Mem-CC into *P. sinuata*. Influence on proline and alanine levels in haemolymph (µmol/ml).

			before	90 min	difference	
			proline			
day 1	Mem-CC	8	86.1 ± 20.3	104.5 ± 22.5	18.4 ± 12.8	**
	H_2O	4	88.8 ± 12.8	89.7 ± 15.4	0.9 ± 10.4	
day 14	Mem-CC	8	94.7 ± 16.3	104.3 ± 19.4	9.6 ± 10.4	*
	H_2O	4	97.4 ± 17.7	101.3 ± 16.8	3.9 ± 7.1	
day 28	Mem-CC	6	68.6 ± 13.1	73.4 ± 11.5	4.8 ± 5.4	
	H_2O	4	72.6 ± 14.0	74.2 ± 11.3	1.0 ± 3.6	
			alanine			
day 1	Mem-CC	8	7.3 ± 4.1	2.3 ± 1.6	-5.0 ± 2.7	**
	H_2O	4	5.7 ± 3.6	5.0 ± 3.4	-0.7 ± 3.0	
day 14	Mem-CC	8	1.5 ± 1.6	0.1 ± 0.2	-1.4 ± 1.4	*
	H_2O	4	0.9 ± 0.6	0.5 ± 0.5	-0.4 ± 0.5	
day 28	Mem-CC	6	0.2 ± 0.4	0.1 ± 0.3	-0.1 ± 0.2	
	H_2O	4	2.3 ± 2.3	2.3 ± 2.3	0.0 ± 0.2	

Significance levels of the difference between resting value and value after the incubation period following inection: * $p < 0.05$, ** $p < 0.005$.

Table 4. Bioassays during starvation experiment. Influence of injection of 50 pmol Mem-CC on total carbohydrate and lipid levels.

		n	before	90 min	difference	
				carbohydrates		
day 1	Mem-CC	8	11.4 ± 4.3	17.9 ± 4.3	6.5 ± 2.1	*
	H_2O	4	10.0 ± 2.3	8.0 ± 2.4	-2.0 ± 1.4	
day 14	Mem-CC	8	1.3 ± 2.3	1.5 ± 2.4	0.2 ± 0.3	
	H_2O	4	1.2 ± 1.0	0.8 ± 1.2	-0.4 ± 0.5	
day 28	Mem-CC	6	1.2 ± 0.6	1.6 ± 0.7	0.4 ± 0.6	
	H_2O	4	1.1 ± 0.5	1 ± 0.3	-0.1 ± 0.3	
				lipids		
day 1	Mem-CC	8	12.8 ± 6.1	10.6 ± 5.2	-2.2 ± 1.1	
	H_2O	4	11.6 ± 3.7	10.1 ± 6.5	-1.5 ± 3.0	
day 14	Mem-CC	8	12.2 ± 11.5	11.5 ± 4.3	-0.7 ± 3.9	
	H_2O	4	8.6 ± 1.6	8.1 ± 1.4	-0.4 ± 1.2	
day 28	Mem-CC	6	14.6 ± 3.7	13.9 ± 4.3	-0.7 ± 3.7	
	H_2O	4	12.2 ± 3.1	11.6 ± 3.2	-0.6 ± 4.2	

Significance levels of the difference between resting value and value after the incubation period following inection: * $p < 0.001$.

3.3.5. Flight experiments during starvation

On day 15 and 30 of starvation some experiments with lift generating flight were conducted to investigate the beetle's ability to fly (Fig. 27). After 15 days of starvation, beetles still flew for 3-6 min with lift and proline concentration in the haemolymph dropping from 91.5 ± 15.8 to 52.6 ± 12.6 µmol/ml during this period and recovering to 79.5 ± 16.3 µmol/ml in 1 h of subsequent rest. Concomitantly, haemolymph alanine concentration increased from 2.0 ± 1.7 to 45.4 ± 14.1 µmol/ml during flight and dropped to 11.0 ± 9.9 µmol/ml after subsequent rest. The sum of the two amino acids was 93.5, 98.0 and 90.5 µmol/ml haemolymph during rest, flight, and subsequent rest, respectively.

Beetles were still able to fly for 3-6 min after 30 days of starvation. During 3-6 min of flight (with lift), haemolymph proline concentration declined from 61.4 ± 12.8 to 35.1 ± 8.8 µmol/ml and was almost re-established to initial values, reaching 54.6 ± 12.5 µmol/ml in 1 h of subsequent rest. The respective alanine concentrations were 0.3 ± 0.5, 26.3 ± 5.8 and 6.7 ± 6.7 µmol/ml. The sum of the two amino acids was 61.7, 61.4 and 61.3 µmol/ml haemolymph during rest, flight, and subsequent rest, respectively.

No change was observed in the carbohydrate and lipid levels, either after flight or subsequent rest following flight after 15 and 30 days of starvation.

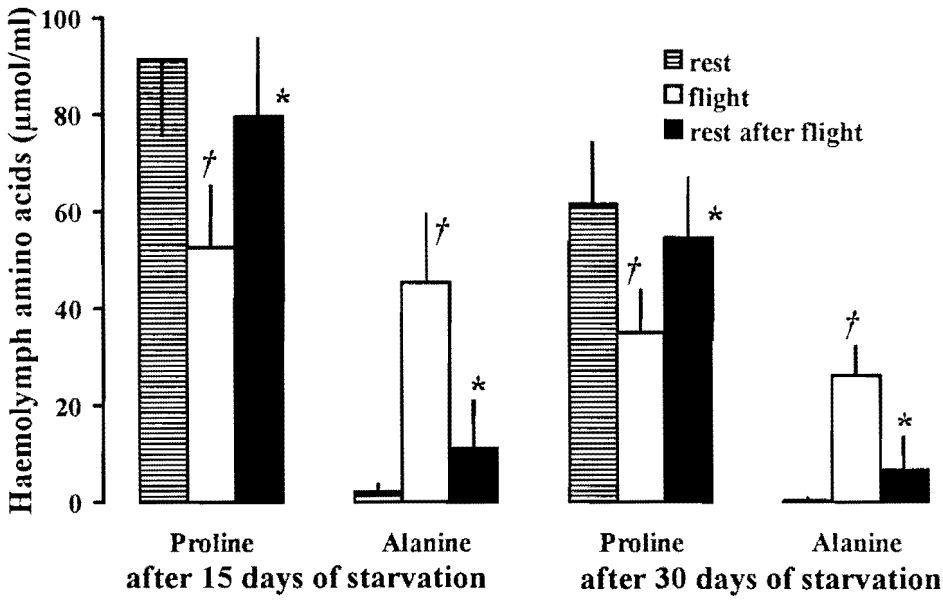

Figure 27. Haemolymph concentration of proline and alanine during rest, 3-6 min of lift generating flight and rest of 60 min after flight in *P. sinuata* which had been starved for different periods. Values are means ± S.D., n = 5. Significance level applied was $p < 0.005$ (†). For the resting period following flight, values were compared with those when flight was terminated. Significance level indicated is: $p < 0.005$ (*).

3.4. Enzyme activities

The maximum activity of enzymes of certain pathways can be used to draw conclusions about the quantitative importance of a particular pathway for flight metabolism (according to Pette; reviewed by Beenakkers *et al.*, 1984). The maximum activities of GluDH, AlaT, AspT, NAD-dependent ME (NAD-ME), NADP-dependent ME (NADP-ME), MDH, GAPDH, HOAD and glycogen phosphorylase a and b were measured in flight muscle and fat body of *P. sinuata* and *D. lunata*, beetles which use a combination of proline and carbohydrates. For comparative reasons, *L. migratoria*, which uses carbohydrates for the first flight period and lipids for extended flight duration, was included in the study (Tables 5 and 6).

3.4.1. Flight muscles

Results of enzyme tests in flight muscle tissue are shown in Table 5.

In *D. lunata*, AlaT has less than 50 % of the activity found in *P. sinuata*, but the activity of GluDH is in the same range (or even higher, when considering that whole thorax preparations of the blister beetle were used). In the locust, however, the two enzymes had much less activity; totalling only 3 and 4 % of values recorded for fruit beetles.

In contrast to AlaT and GluDH, the activity of another enzyme of amino acid metabolism,

AspT, was at the same low level in both the fruit beetle and in the locust.

The activity of MDH was high in all three insects investigated. However, the highest activity was recorded from *P. sinuata* flight muscles, while those of *L. migratoria* and *D. lunata* had only 72 and 40 % of the values observed for *P. sinuata*.

A high value for the activity of NAD-dependent ME was measured in *P. sinuata*, with the blister beetle also having high activity, with 74 % of activity recorded in the fruit beetle. In locusts, however, ME activity was only 17 % of that found in *P. sinuata*.

NADP-ME of *P. sinuata* appeared to be less important than NAD-ME and had only a third of the activity of the latter. In the locust, however, importance of NAD-ME was relatively lower than in the beetle, while the activity of NADP-ME was negligible.

The maximum activity of a key enzyme in the β-oxidation of fatty acids, HOAD, is an indication of the direct oxidation of lipids to power flight. The activity of HOAD in *P. sinuata* flight muscles was 22 % of that found in the flight muscles of locusts. However, this activity was three-times that of the blister beetle, where HOAD activity was very low.

The maximum activity of glycogen phosphorylase in the flight muscles demonstrates its ability to break down glycogen for the supply of energy via the glycolytic pathway. The activity of phosphorylase a in the flight muscles of *P. sinuata* was about 41 % of that measured in *L. migratoria* and was activated 7.5-fold in the beetle, while in flight muscles of the locust activation was only 2.5-fold.

An enzyme of the glycolytic pathway, GAPDH, had a high activity in *P. sinuata*, which was in the same range in *L. migratoria* and *D. lunata*.

Table 5. Maximum enzyme activities (U/gfw) in flight muscle tissue of *P. sinuata*, *D. lunata* and *L. migratoria*.

	n	*P. sinuata*	n	*L. migratoria*	n	*D. lunata*
AlaT	6	195.2 ± 23.5	4	5.4 ± 0.9	5	92.1 ± 11.7
AspT	7	10.4 ± 2.1	4	16.1 ± 1.2		not measured
GluDH	6	107.7 ± 10.1	4	4.7 ± 0.2	5	117.5 ± 9.7
MDH	6	321.4 ± 38.9	4	230.2 ± 28.9	5	127.9 ± 18.8
ME-NAD	6	24.2 ± 1.9	5	4.2 ± 1.0	5	17.9 ± 3.3
ME-NADP	6	8.1 ± 1.1	5	1.1 ± 0.2		not measured
HOAD	6	18.9 ± 2.3	5	84.2 ± 8.9	5	6.0 ± 2.9
GAPDH	6	254.5 ± 27.0	5	178.9 ± 21.1	4	224.8 ± 10.0
Phosphorylase *a*	6	5.0 ± 1.1	5	5.7 ± 1.3		not measured
Phosphorylase *a* + *b*	6	42.3 ± 3.1	5	14.2 ± 2.7		not measured

Values are expressed as units per gfw, as means ± S.D.
1 unit of enzyme activity = 1 µmol substrate used per min.

3.4.2. Fat body

Results of enzyme tests in fat body tissue are shown in Table 6.

AlaT had a 30-fold higher activity in *P. sinuata* than in *L. migratoria*, while the activity of AspT was in the same low range in both insects. Maximum activity of GluDH was almost negligible in the fat bodies of both animals.

The activity of NAD-ME was negligible in the fat body of beetles and locusts, while the activity of NADP-ME in *P. sinuata* fat body was about 50 % of that of NAD-ME in the beetle's flight muscle. In *L. migratoria*, however, activity of NADP-ME in fat body was as low as in the flight muscle.

MDH activities in the fat bodies of the beetle and the locust were low in comparison with their flight muscles (4 % and 31 %, respectively).

HOAD in the fat body of *P. sinuata* had a 4.4-fold higher maximum activity compared with the locust but was in the same range of activity as in the beetle's flight muscles.

Activities of phosphorylase *a* were similar in the fat bodies of both insects in comparison to their respective flight muscle values. However, the enzyme was activated 5.3-fold in the beetle, while it was only activated 2.7-fold in the locust. These ratios are similar to the ones obtained for the activation rates of the glycogen phosphorylase in flight muscles of the two insects.

Although the activity of the GAPDH in the fat bodies of both animals was in the same range, it was substantially less than that in their respective flight muscles. Whereas in *P. sinuata* the activity was 20 % of the flight muscle value, it was only 13 % in locust flight muscles compared with activities in their fat body.

Table 6. Maximum enzyme activities (U/gfw) in fat body tissue of *P. sinuata* and *L. migratoria*.

	n	*P. sinuata*	n	*L. migratoria*
AlaT	7	133.5 ± 12.1	4	4.4 ± 0.5
AspT	7	4.6 ± 0.7	4	6.9 ± 0.4
GluDH	7	1.1 ± 0.06	5	0.8 ± 0.2
MDH	7	12.8 ± 1.0	5	72.2 ± 5.1
ME-NAD	6	0.3 ± 0.03	5	0.04 ± 0.01
ME-NADP	7	11.3 ± 0.5	5	1.6 ± 0.1
HOAD	5	21 ± 4.2	5	4.8 ± 0.3
GAPDH	6	52.1 ± 3.9	5	23.5 ± 2.1
Phosphorylase *a*	6	4.0 ± 0.5	5	10.0 ± 2.6
Phosphorylase *a* + *b*	6	21.0 ± 1.8	5	26.8 ± 1.9

Data are given as means ± S.D., as units/gfw (see previous table).

3.5. Mitochondria experiments

Table 7 shows that flight muscle mitochondria of *P. sinuata* favoured the oxidation of proline and pyruvate (in the presence of ADP and phosphate → state 3; see 2.8.2.). Respiratory control rates were high for both substrates and the P/O values approached the theoretical maximum of 3 for NADH oxidation. The relatively high values for the oxidation of α-glycerophosphate both in the presence and absence of Ca^{2+}, indicates that a functioning glycerophosphate shuttle was present. The other substrates tested had much lower oxidation rates. While proline seems to be the preferred substrate to pyruvate in *P. sinuata*, pyruvate is oxidised at about double the rate of proline in locusts. The glycerophosphate shuttle seemed to be more active in locusts than in the beetles. Despite the fact that lipid concentration increased with flight duration, it was not possible to show substantial respiration of the beetle's flight muscle mitochondria with palmitoyl-carnitine.

Table 7. Oxygen consumption of flight muscle mitochondria of *P. sinuata* and several other insects with different substrates.

		μg - atom O_2/min x mg protein					
	substrate (mM)	*P. sinuata*	*L. migratoria*	*D. lunata**	*T. fascicularis*	*Lepithrix spec.**	*C. innocua**
30	proline	4.01 ± 0.09	0.99 ± 0.21	1.77 ± 0.47	3.84 ± 1.11	4.13 ± 0.73	3.14 ± 0.40
1	pyruvate	2.81 ± 0.82	1.82 ± 0.63	2.95 ± 0.98	2.64 ± 0.94	3.25 ± 0.92	3.01 ± 1.10
20	α - glycerophosphate	0.45 ± 0.11	1.53 ± 0.41	0.38 ± 0.02	0.21 ± 0.10	0.22 ± 0.11	0.47 ± 0.05
20	α - glycerophosphate + Ca^{2+}	0.91 ± 0.12	not measured	0.94 ± 0.03	0.62 ± 0.10	0.53 ± 0.21	0.97 ± 0.20
10	glutamate	0.12 ± 0.03	not measured	0.18 ± 0.01	0.14 ± 0.02	0.30 ± 0.30	0.17 ± 0.05
10	aspartate	0.08 ± 0.01	not measured	0.00 ± 0.00	0.03 ± 0.02	0.09 ± 0.09	0.08 ± 0.01
10	malate	0.10 ± 0.02	not measured	0.10 ± 0.01	0.10 ± 0.02	0.07 ± 0.07	0.04 ± 0.01
10	alanine	0.07 ± 0.01	not measured	0.10 ± 0.01	0.12 ± 0.03	0.09 ± 0.09	0.07 ± 0.01
10	citrate	0.03 ± 0.02	not measured	0.00 ± 0.00	0.02 ± 0.01	0.01 ± 0.01	0.03 ± 0.01
10	oxoglutarate	0.09 ± 0.04	not measured	0.02 ± 0.02	0.10 ± 0.04	0.09 ± 0.09	0.08 ± 0.02
0.2 10	palmitoyl-carnitine + malate	0.19 ± 0.02	2.11 ± 0.32	not measured	not measured	not measured	not measured
	n	5 - 10	4 - 5	3 - 5	4	4	4
		proline	pyruvate				
	P/O ratio for *P. sinuata*	2.6 - 3.0	2.4 - 2.8				
	RCR	21 - 120	24 - 38				

Values are means ± S.D., (*) each sample consisted of flight muscles of 5 animals.
Rates were measured during state 3 respiration achieved by the presence of ADP (1.5 mM).

The data obtained from respiration experiments with flight muscle mitochondria of the four other beetle species (see Table 7) resembled the observations for *P. sinuata* flight muscles. Proline and pyruvate caused the highest respiration rates. While in *T. fascicularis, Lepithrix* sp. and *C. innocua* proline was favoured, in *D. lunata* pyruvate led to the highest oxygen consumption by flight muscle mitochondria. In all four species, α-glycerophosphate was consumed at moderate rates which could be increased by the addition of Ca^{2+}. All other substrates were not oxidised or caused negligible respiration

rates.

Because carbohydrates and proline are broken down for the supply of energy to the flight muscles during flight and because pyruvate and proline are oxidised at high rates by *P. sinuata* flight muscle mitochondria, it was investigated whether co-oxidation causes an additive respiration rate. From Table 8 it can be concluded that the rates are additive only at low concentrations of proline. At higher proline concentrations, however, the rate was lower than expected. At very high proline concentrations, the co-oxidation rate approaches that of the respective proline oxidation.

Table 8. Co-oxidation of proline and pyruvate by flight muscle mitochondria of *P. sinuata.*

substrate (mM)	µg - atom O_2/min x mg protein	expected	Δ
1 pyruvate	2.03 ± 0.12		
0.5 proline	0.36 ± 0.05		
0.5 proline + 1 pyruvate	2.47 ± 0.31	2.39	0.08
1 proline	0.53 ± 0.07		
1 proline + 1 pyruvate	2.57 ± 0.41	2.56	-0.01
1.5 proline	0.76 ± 0.11		
1.5 proline + 1 pyruvate	2.29 ± 0.16	2.79	-0.5
2 proline	0.91 ± 0.12		
2 proline + 1 pyruvate	2.37 ± 0.35	2.94	-0.57
5 proline	1.71 ± 0.32		
5 proline + 1 pyruvate	2.56 ± 0.18	3.74	-1.35
10 proline	2.67 ± 0.61		
10 proline +1 pyruvate	2.99 ± 0.48	4.7	-1.7

Values were measured during state 3 respiration (see previous table).
Results are given as means ± S.D.
Δ is the difference between the measured value of co - oxidation and the value obtained by adding the values of pyruvate and proline oxidation (measured separately).

Table 7 shows that the oxidation of proline as well as pyruvate is tightly coupled to the respiratory chain and strongly controlled by ADP availability in flight muscle mitochondria of *P. sinuata*. Because ADP can also control substrate oxidation at sites other than the respiratory chain (Sacktor, 1975), the dependence of proline and pyruvate oxidation on ADP was investigated in more detail. Additionally, the substrate dependence of oxidation was examined. These data can be helpful in explaining regulatory mechanisms such as the co-oxidation of the two substrates (Table 8) and their relevance *in vivo*.

Figure 28 depicts the relationship between proline oxidation and ADP

concentration. The K_M value, calculated by means of the Lineweaver-Burk-plot, is 0.098 mM, while the K_M value of pyruvate oxidation with different ADP concentrations (Fig. 29) is 0.125 mM. Figure 30 illustrates the substrate dependence of state 3 respiration with proline. From these data a K_M of 6.6 mM could be calculated. There were no problems in demonstrating pyruvate oxidation without malate as a primer as reported by de Kort *et al.* (1973) for the Colorado beetle and a relationship of state 3 respiration and substrate concentration was found (Fig. 31). The calculated K_M was 0.54 mM.

An experiment was conducted to determine the stoichiometry of proline oxidation and alanine production by isolated flight muscle mitochondria. Samples for amino acid determination were drawn at two different times when the oxygen consumption of the sample was constant and the difference between the two samples was used to calculate proline consumption, alanine production and oxygen consumption of the respective flight muscle preparation. While state 3 respiration in the presence of 1.5 mM ADP was 1.84 ± 0.11 µmol O_2/min x mg protein (3.68 ± 0.22 µg/min x µg protein), 0.76 ± 0.08 µmol proline/min/µg protein was consumed and 0.80 ± 0.07 µmol alanine formed (n = 8). The difference between the amount of proline oxidised and the amount of alanine generated was not significantly different.

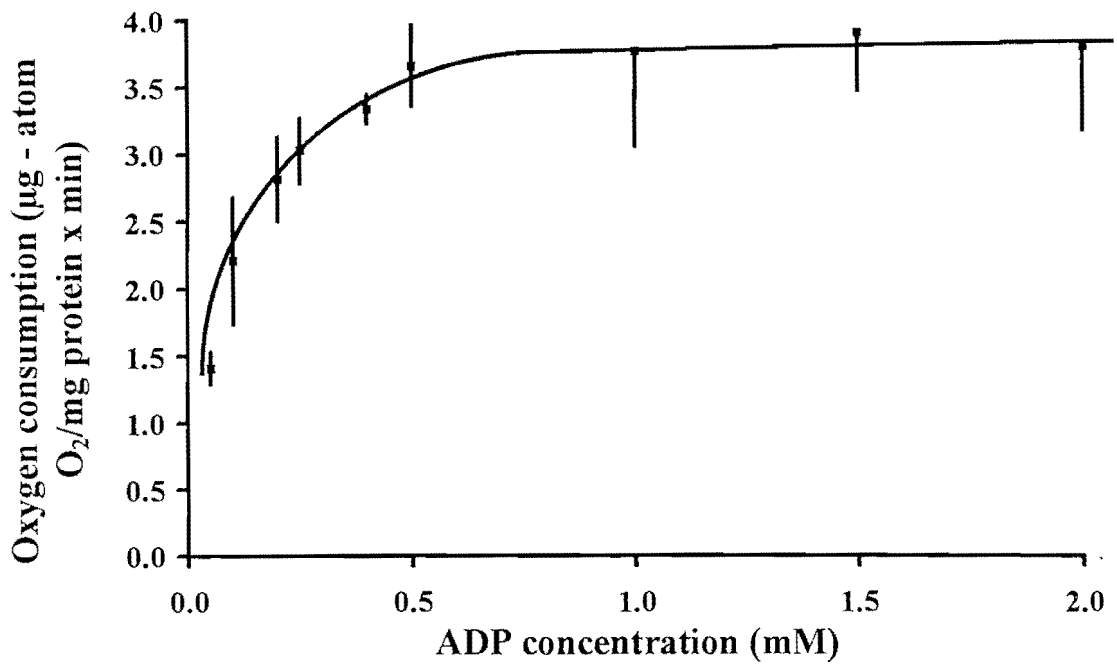

Figure 28. Relationship between proline oxidation (30 mM final concentration) and ADP concentration of flight muscle mitochondria of *P. simuata*. Values are given as means ± S.D., n = 4-5.

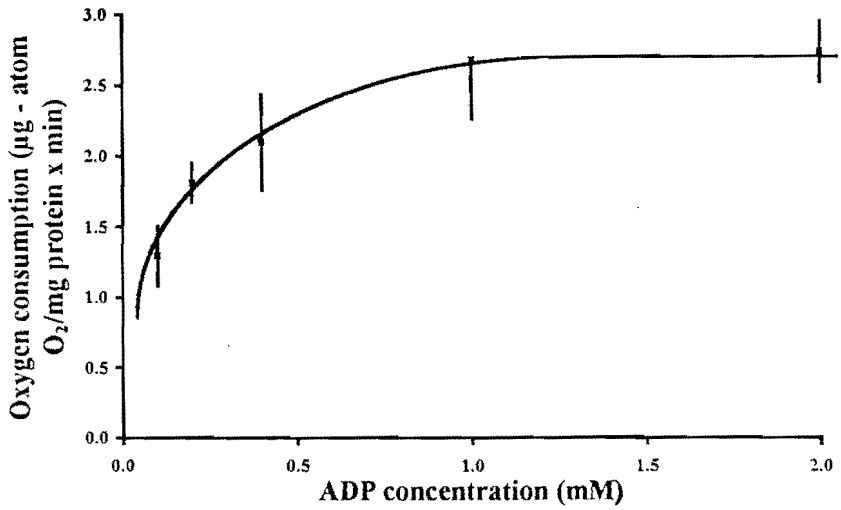

Figure 29. Relationship between pyruvate oxidation (1 mM final concentration) and ADP concentration of flight muscle mitochondria of *P. simuata*. Values are given as means ± S.D., n = 3-5.

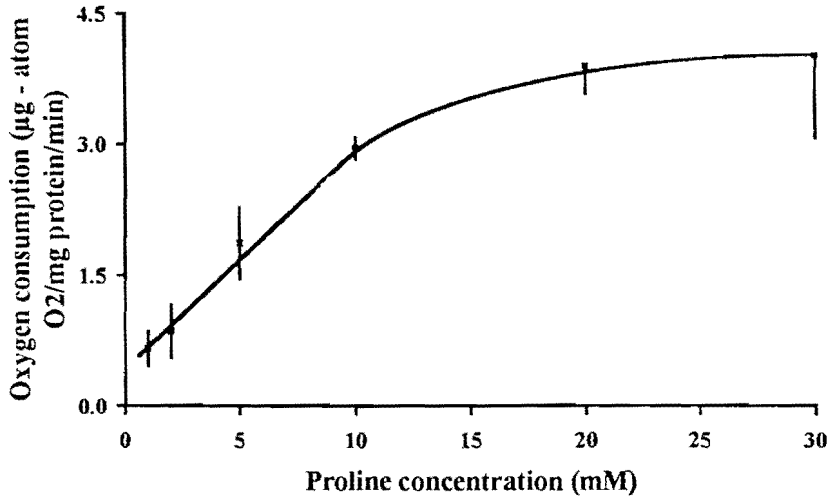

Figure 30. Substrate dependence with proline during state 3 respiration (in the presence of 1.5 mM ADP) of flight muscle mitochondria isolated from *P. simuata*. Values are given as means ± S.D., n = 5-10.

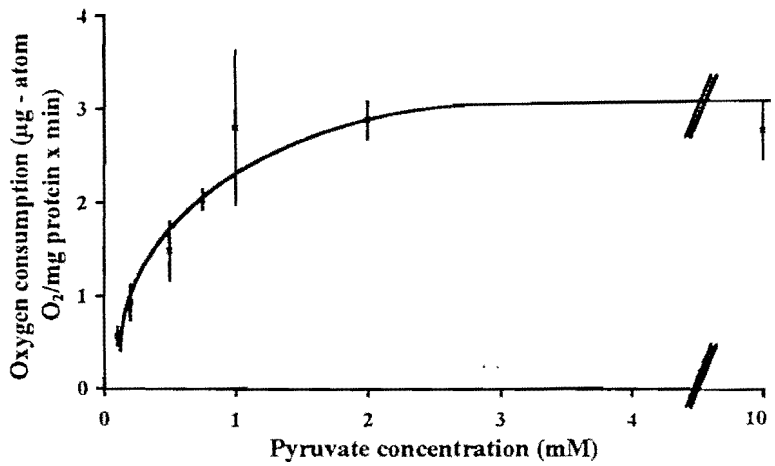

Figure 31. Substrate dependence with pyruvate during state 3 respiration (in the presence of 1.5 mM ADP) of flight muscle mitochondria isolated from *P. simuata*. Values are given as means ± S.D., n = 5-10.

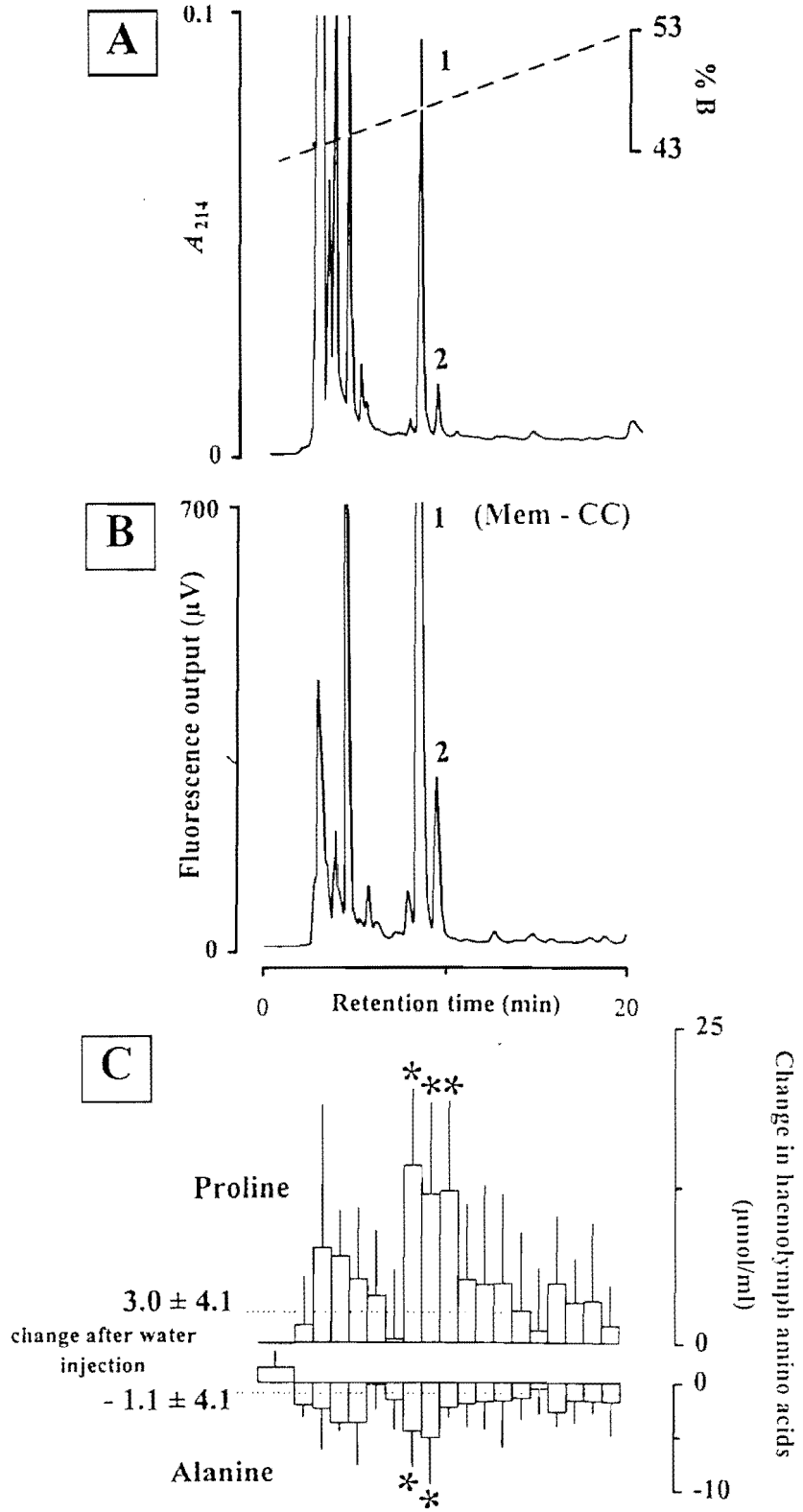

Figure 32. An extract of 18 corpora cardiaca from *P. sinuata* was run on HPLC and the UV absorbence (A) and the fluorescence signal (B) recorded. One minute fractions were collected, and after drying and re-dissolving, 1.5 gland equivalents were injected in a conspecific bioassay (n = 8-10). The change of proline and alanine concentrations was determined from haemolymph samples which were taken before injection and 90 min thereafter (C). Values are means ± S.D. *Significantly different in paired t-test (at least $p < 0.01$) and t-test compared with water injected group (at least $p < 0.05$).

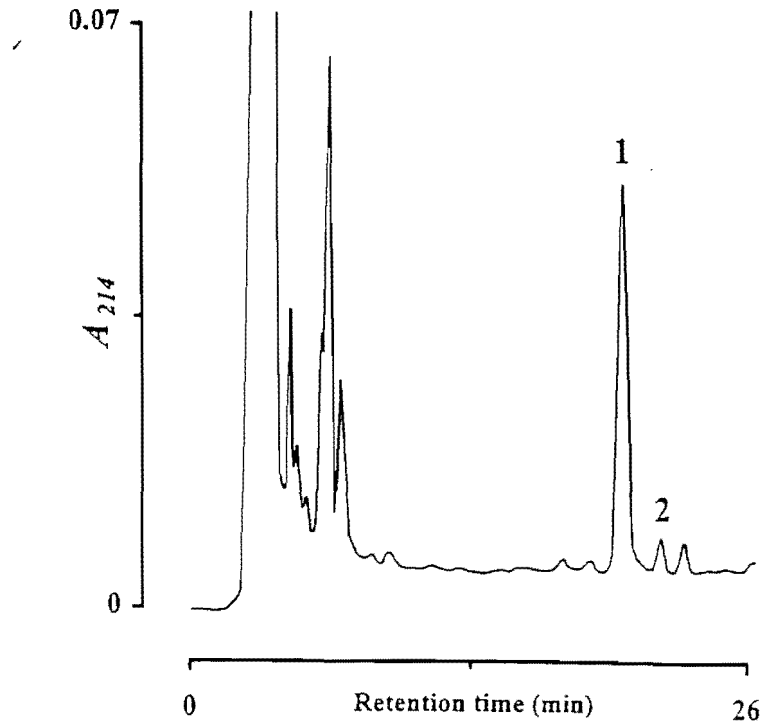

Figure 33. UV trace of an HPLC run (33-53 % B in 40 min) of a methanolic pCC extract of *P. sinuata* glands (20 pCC). The peak material was collected and used in a conspecific bioassay (see Table 10).

A better separation of the peaks labelled 1 and 2 in Figure 32 was obtained when an extract from 20 glands was run on RP-HPLC, employing a shallower gradient (Fig. 33). Material of these peaks(1.5 pCC-equivalents), labelled 1 and 2, were injected into *P. sinuata* (Table 10). Both peaks caused a significant increase in the proline level and a significant decrease in the alanine level, which was similar to the results obtained after injection of 50 pmol of synthetic Mem-CC. No significant changes were observed after injection of distilled water as a negative control.

Table 10. Changes in haemolymph amino acid concentrations in *Pachnoda sinuata* after injection of peak material of 1.5 pCC-equivalents (in 10 µl) of *P. sinuata*.

	n	0 min	90 min	difference		
			Proline (µmol/ml)			
H_2O	8	97.1 ± 3.5	93.0 ± 13.6	-4.1 ± 12.5		
peak 1	10	96.3 ± 8.2	108.3 ± 8.8	12.0 ± 5.5	†††	**
peak 2	13	96.4 ± 8.5	104.0 ± 9.4	7.6 ± 3.6	†††	*
			Alanine (µmol/ml)			
H_2O	8	11.4 ± 4.6	11.0 ± 1.9	-0.4 ± 4.3		
peak 1	10	8.7 ± 4.1	1.0 ± 0.4	-7.7 ± 1.7	†††	***
peak 2	13	7.5 ± 2.7	3.3 ± 3.1	-4.1 ± 1.4	†††	*

Significance levels for differences in paired t-test: $p<0.05$ (†), $p<0.02$ (††), $p< 0.001$ (†††).
Significances for differences compared with value of dH_2O injection:
$p<0.05$ (*), $p<0.02$ (**), $p<0.01$ (***).

Because intact beetles had been used for bioassays, an experiment was conducted, where the source of the neuropeptides was excluded by decapitation of the beetles. In a pilot experiment, concentration of metabolites were measured during each of three days after decapitation (Fig. 34).

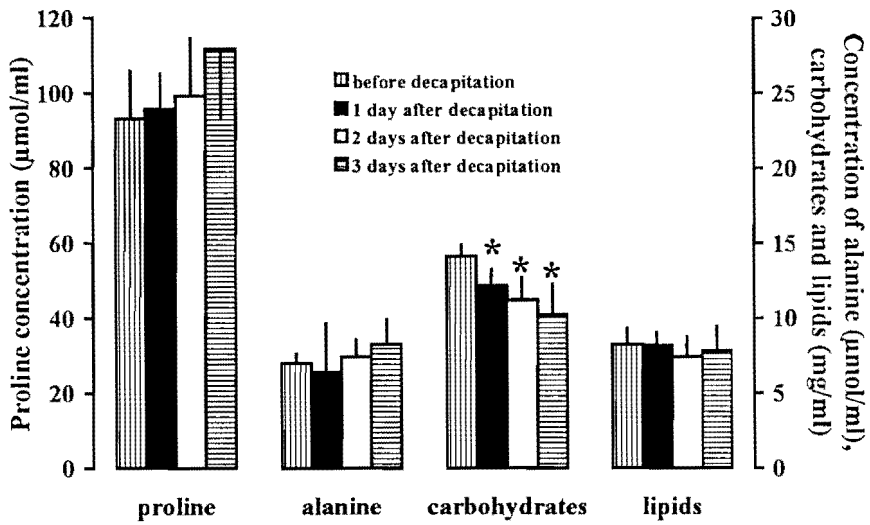

Figure 34. Metabolite concentrations in the haemolymph of *P. sinuata* before and various days after decapitation. Values are given as means ± S.D., n = 4. Significance level of the difference compared with the values of the intact beetles * $p < 0.02$.

Table 11. Changes in haemolymph metabolites after injection of Mem - CC into *P. sinuata* 1 day after decapitation.

	Treatment		
	Control (10 µl of distilled water)	50 pmol Mem-CC	
	proline (µmol/ml)		
0 min	96.3 ± 11.8	98.4 ± 8.0	
120 min	95.8 ± 12.9	120.6 ± 11.6	
difference	-0.5 ± 2.4	22.2 ± 7.3	††
n	6	6	
	alanine (µmol/ml)		
0 min	2.4 ± 2.2	2.5 ± 2.1	
120 min	1.8 ± 2.5	0.2 ± 0.1	
difference	-0.6 ± 1.7	-2.3 ± 2.1	†
n	6	6	
	lipids (mg/ml)		
0 min	7.1 ± 2.5	7.4 ± 2.4	
120 min	7.2 ± 2.6	7.3 ± 2.4	
difference	0.1 ± 0.4	-0.1 ± 0.6	
n	5	6	
	carbohydrates (mg/ml)		
0 min	7.3 ± 2.8	9.0 ± 2.5	
120 min	7.6 ± 3.5	15.9 ± 3.2	
difference	0.3 ± 0.9	6.9 ± 1.9	††
n	11	11	

Values are means ± S.D.
The significance of the differences before and after injection is indicated by † $p < 0.05$ and †† $p < 0.001$, using paired t test.

From these data it was decided to perform the bioassays one day after decapitation because levels of proline, alanine and lipids were not significantly different to those of the beetles before decapitation, while the change in the concentration of carbohydrates was still small (Fig. 34). Therefore, bioassays were carried out with decapitated beetles (Table 11). Similar to the observation with non-decapitated beetles, haemolymph proline concentration rose, while alanine concentration dropped after injection of Mem-CC (Table 11). No changes were observed in the control group which was injected with distilled water. While lipid levels remained unchanged in both groups, a significant increase in haemolymph carbohydrates was found after injection of Mem-CC (Table 11).

The increase of the proline concentration following injection of bioactive material is relatively small when compared with the adipokinetic effect of AKH peptides in locusts and their hypertrehalosaemic effect in cockroaches. Because this may have been caused by a lack of alanine at rest (the precursor for re-synthesis of proline), either crude corpus cardiacum extract, alanine, crude corpus cardiacum extract plus alanine or 50 pmol Mem-CC were injected, and the time-dependence of the response recorded (Fig. 35). In each experiment a time-dependent increase in haemolymph proline levels and a time-dependent decrease in haemolymph alanine levels were measured. Maximum proline concentration was reached between 60 and 120 min of incubation, while alanine concentration dropped consistently. However, co-injection of corpus cardiacum extract plus alanine did not increase proline concentration further than could be achieved with the injection of either corpus cardiacum extract or Mem-CC alone. Interestingly, injection of alanine was sufficient to cause a rise in the proline concentration. Control injections of water, however, did not result in any changes in the concentrations of proline and alanine during the entire duration of the experiment.

Another factor which could limit the maximum possible increase of the proline concentration may be the high resting level of proline itself, resulting in a feedback inhibition. Therefore, the relationship of the increase in the proline level and the resting value of proline was analysed. Because the resting concentrations of alanine, the precursor for the re-synthesis of proline in the fat body (see section 3.8.) were usually low, the relationship between the resting alanine concentration and the response of proline concentration upon injection of corpora cardiaca material was also investigated. Values from a number of conspecific bioassays with injection of 1 pCC equivalent of *P. sinuata* were used for constructing Figure 36. These data show a negative correlation of the

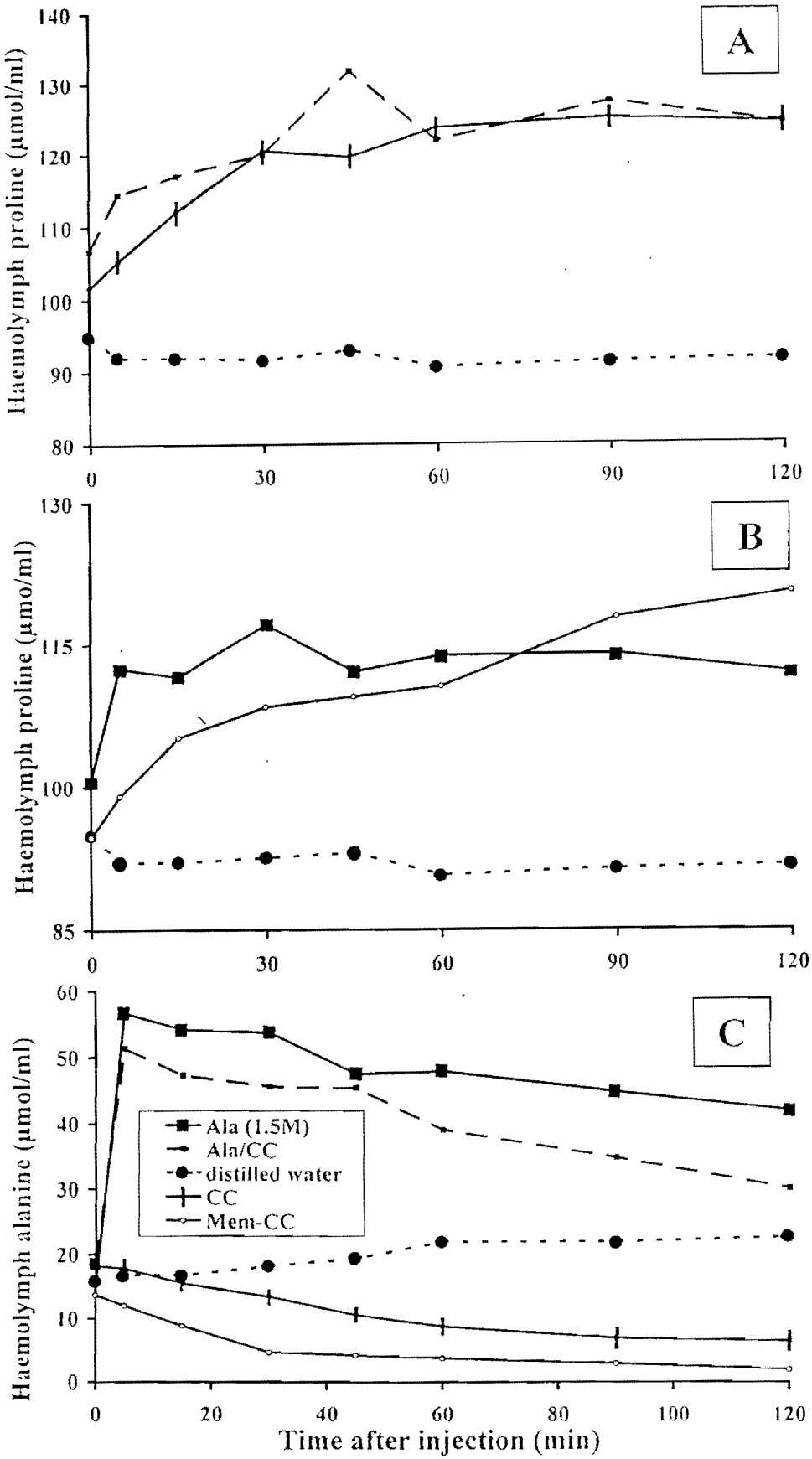

Figure 35. Time course of haemolymph proline (A, B) and alanine (C) concentration of *P. simuata* after injection of various substances. One pair of corpora cardiaca and 50 pmol Mem-CC were injected. Values are means of at least 5 individuals. S.D. is not shown for clarity but was always less than 30% of the mean.

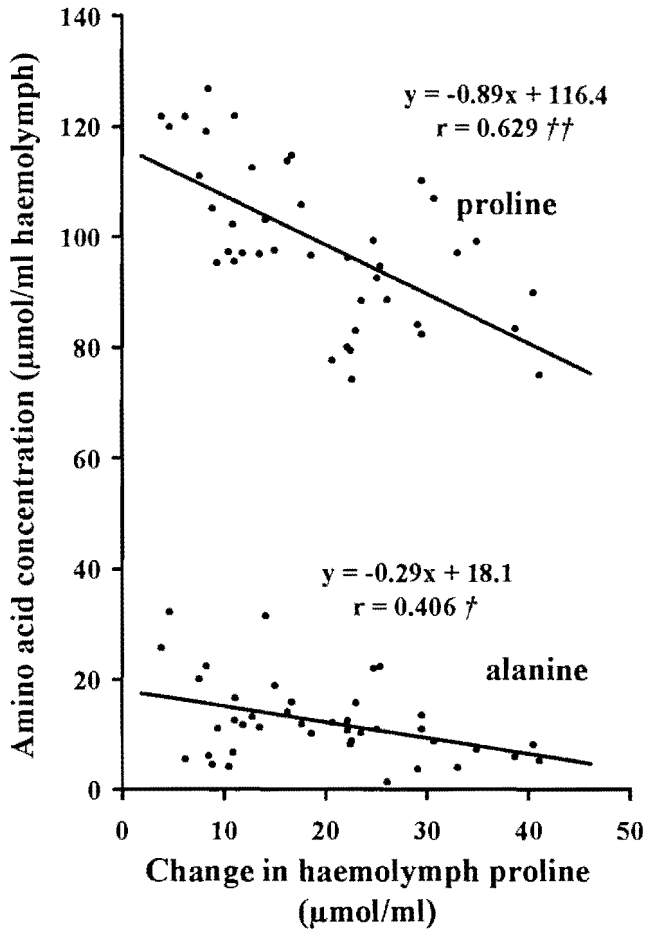

Figure 36. Relationship between the pre–injection concentration of proline and alanine and the post–injection increase of proline levels after conspecific injection of crude *P. sinuata* corpus cardiacum extract. Values were taken from 41 injections of different experiments. Significance of correlation † $p < 0.01$ and †† $p < 0.001$.

resting proline concentration and the value of change. Surprisingly, a negative correlation was also recorded with the resting alanine concentration. This confirms that although alanine is a substrate for the re-synthesis of proline, alanine concentration in the haemolymph was not essential for the increase in proline level.

For investigating dose-response relationships, a metabolic situation in *P. sinuata* was needed where the haemolymph proline concentration was relatively low in order to attain higher increases after injection of active material. Because starvation of up to four weeks did not lead to such a decrease of the proline haemolymph concentration (see section 3.3.1.), the concentrations of proline and alanine in the haemolymph were measured at different times of the day to find out whether they followed a certain time-course pattern. Although proline concentrations had different levels on two consecutive days, a clear circadian rhythm was found with a maximum of 126.5 ± 16.4 µmol/m at

5:00 and a minimum of 82.3 ± 19.1 µmol/ml at 22:00 (Fig. 37). Therefore, experiments of which the results are shown in Figure 38 and 39, were carried out at 22:00 (shortly after lights off). Because these bioassays are time-consuming, the number of animals in each experimental group was kept small to exclude an influence of the "naturally" occurring increase of the proline level as time progressed (the 'window' of low proline concentration is between 22:00 and 1:00). The concentration of alanine was rather random throughout the day and was not related to the time-course of the haemolymph proline. However, the differences in individual alanine concentration of the beetles were much smaller at the time of the proline minimum (results not shown in Fig 37).

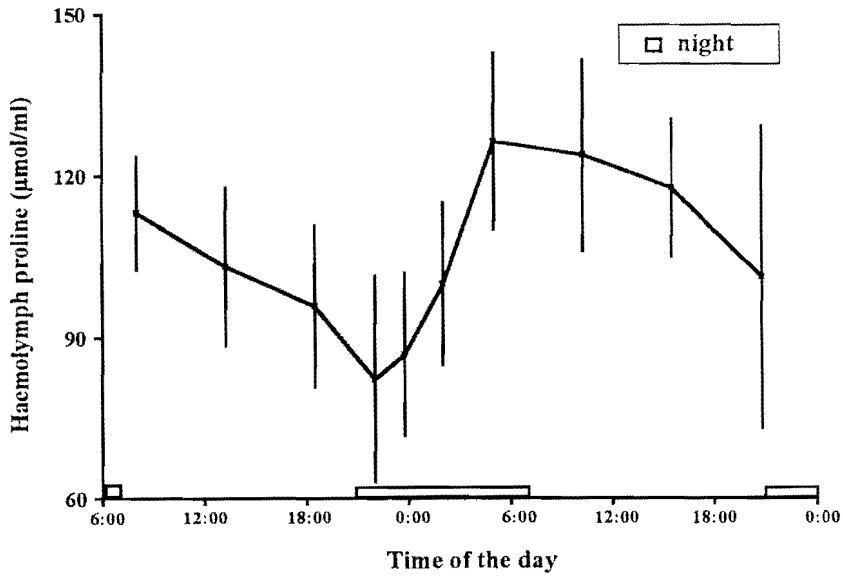

Figure 37. Circadian course of haemolymph proline concentration of *P. simuata*. Values are means and S.D. (n = 8).

This circadian course of proline concentration might be regulated by Mem-CC, to examine this an experiment was conducted where one group of beetles was ligated by means of a thin thread around the neck. Haemolymph of two groups of six animals was taken at 8:00 am. Six beetles (group A) were subsequently ligated (see above). The haemolymph concentration of those beetles was 113.1 ± 2.6 µmol/ml and that of alanine 8.2 ± 2.1 µmol/ml. Beetles of the non-ligated control group (B) had a haemolymph proline level of 114.6 ± 11.0 µmol/ml and an alanine level of 7.1 ± 1.4 µmol/ml. The respective values of the two groups were not significantly different from each other. Twenty-four hours later, a second sample was taken from each beetle. The haemolymph concentrations were as follows: group A, proline 87.4 ± 13.0 µmol/ml, alanine 7.2 ± 1.4 µmol/ml; group B, proline 117.8 ± 2.8 µmol/ml, alanine 8.1 ± 2.2 µmol/ml. The

haemolymph proline concentrations of the two groups were significantly different from each other ($p < 0.001$), while the difference of the alanine levels was insignificant. The proline concentrations of animals of group A before and after ligation were significantly different ($p < 0.001$).

A clear dose-response relationship was established for the concentration of Mem-CC injected and the increase of the proline haemolymph concentration (Fig. 38A). The ED_{50} was calculated to be 1.41 pmol. The maximum response of around 23 µmol/ml was achieved with about 25 pmol of Mem-CC. The decrease in haemolymph alanine was also dose-dependent (Fig. 38B). The ED_{50} was 0.88 pmol and ED_{max} 15 pmol.

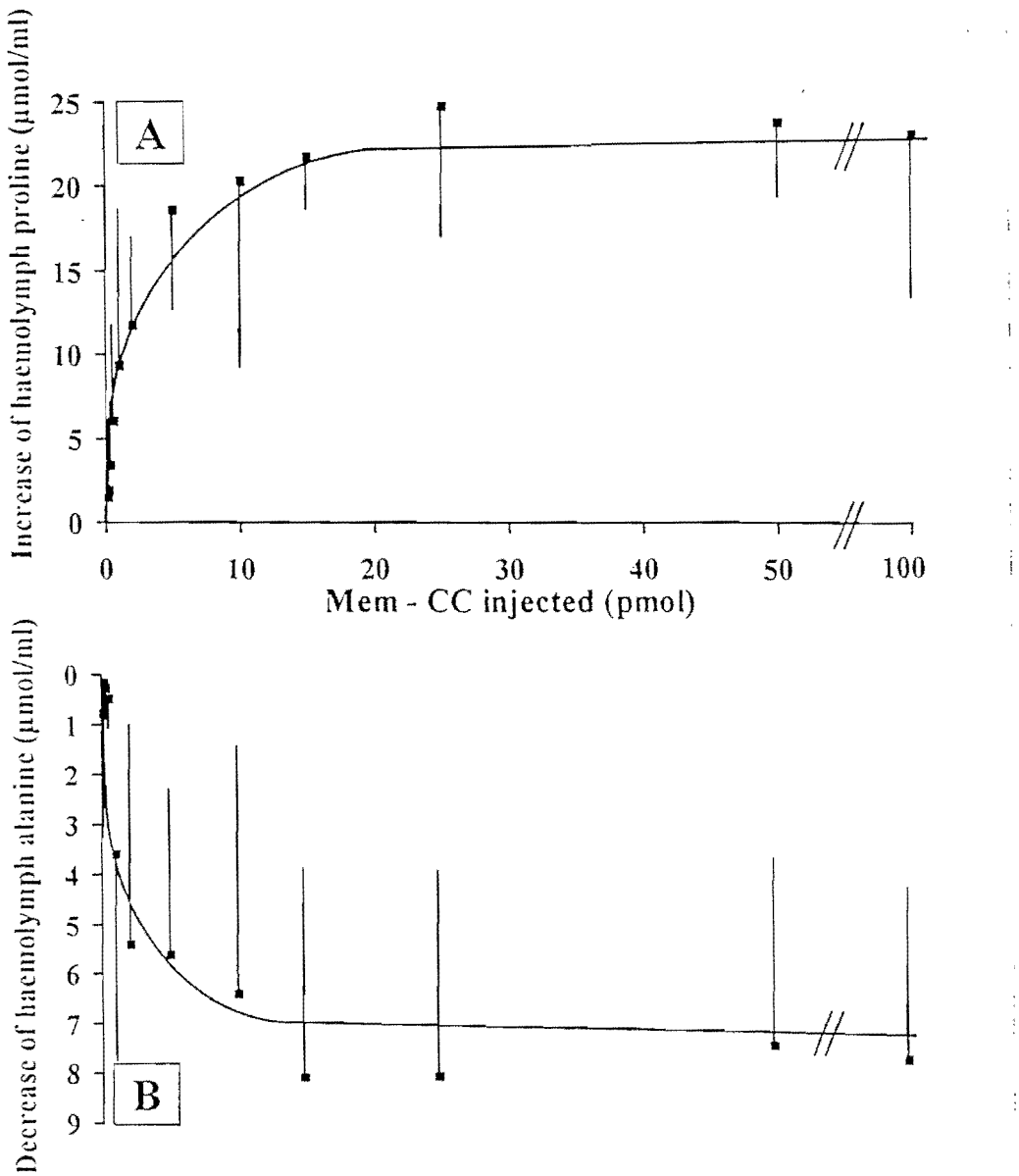

Figure 38. Dose response relationship of the influence of injected Mem-CC on haemolymph proline (A) and alanine (B) concentrations of *P. sinuata*. Values are means ± S.D. (n = 7-16).

Table 12. Changes in haemolymph proline and alanine concentration after injection of 50 pmol of several members of the AKH/RPCH peptide family.

	Sequence (position)										Change in amino acid concentration 90 min after injection (µmol/ml haemolymph)					
injected	1	2	3	4	5	6	7	8	9	10	n	**Proline**			**Alanine**	
dH_2O											29	3.1 ± 10.8			-0.3 ± 2.5	
Mem-CC	pGlu	Leu	Asn	Tyr	Ser	Pro	Asp	Trp			18	16.6 ± 7.6	****	††††	-4.4 ± 2.6	**** ††††
Del-CC	pGlu	Leu	Asn	Phe	Ser	Pro	Asn	Trp	Gly	Asn	16	16.2 ± 7.9	****	††††	-2.7 ± 2.6	**** †††
Scd-CC-II	pGlu	Phe	Asn	Tyr	Ser	Pro	Val	Trp			20	2.7 ± 11.6			-1.9 ± 3.4	*
Ona-CC	pGlu	Tyr	Asn	Phe	Ser	Thr	Gly	Trp			25	9.7 ± 8.4	****	†	-3.4 ± 3.0	**** ††††
Pea-CAH-II	pGlu	Leu	Thr	Phe	Thr	Pro	Asn	Trp			15	17.1 ± 14.3	****	††††	-1.9 ± 2.3	**
Lom-AKH-III	pGlu	Leu	Asn	Phe	Thr	Pro	Trp	Trp			10	-0.4 ± 13.3			-0.1 ± 2.2	
Scd-CC-I	pGlu	Phe	Asn	Tyr	Ser	Pro	Asp	Trp			10	13.1 ± 5.0	****	††	-4.7 ± 2.5	**** ††††
Mem-Analogue	pGlu	Leu	Asn	Tyr	Ser	Pro	Asn	Trp			10	11.0 ± 7.9	***	†	-4.9 ± 3.4	*** ††††
Tem-HrTH	pGlu	Leu	Asn	Phe	Ser	Pro	Asn	Trp			13	11.0 ± 8.8	****	†	-2.0 ± 1.6	**** †
Pht-HrTH	pGlu	Leu	Thr	Phe	Ser	Pro	Asp	Trp			8	15.0 ± 5.3	****	††	-1.5 ± 1.7	*
Ani-AKH	pGlu	Leu	Thr	Phe	Ser	Pro	Ser	Trp			10	1.7 ± 20.5			-0.9 ± 1.9	
Pea-CAH-I	pGlu	Val	Asn	Phe	Ser	Pro	Asn	Trp			8	22.3 ± 12.5	***	††††	-3.0 ± 3.4	* †

Values are means ± SD.

Significance levels: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.002$, **** $p < 0.001$. Paired t-test was used.

T-test of change compared to result of injection of water: † $p < 0.05$, †† $p < 0.01$, ††† $p < 0.005$, †††† $p < 0.001$.

3.6.2. Influence of related AKH peptides and free amino acids on proline metabolism

Information about the structural requirements of the AKH peptides for receptor recognition can be obtained by structure-activity studies. Therefore, bioassays were conducted in which AKH peptides of Coleoptera, other than Mem-CC, and naturally occurring and artificial analogues of Mem-CC were injected into *P. sinuata*. The results are shown in Table 12. Most of the injected AKH - octapeptides elicited a response, except Lom-AKH-III and Scd-CC-II, which had no effect at all on haemolymph proline and alanine concentrations. The activity of Ona-CC was small on elevating proline concentration, but still significantly different from the water injection. A decapeptide (Del-CC) was equally active as many of the octapeptides tested.

Table 13. Changes of haemolymph proline and alanine concentration after injection of 1 M free amino acids (in 10 µl) into *P. sinuata*.

		Change in amino acid concentration after 90 min (µmol/ml haemolymph)	
10 µl injected of	n	**proline**	**alanine**
dH_2O	7	1.7 ± 3.2	0.5 ± 2.5
Ala	5	14.3 ± 6.1 †	
Asp	5	6.1 ± 5.2	-0.1 ± 1.3
Arg	5	4.2 ± 6.3	-1.0 ± 2.1
Glu	5	7.8 ± 8.0	-0.6 ± 1.1
Gly	5	6.2 ± 6.3	-1.9 ± 2.5
Leu	5	3.9 ± 7.8	-0.8 ± 1.2

Values are means ± SD.
Significance level for differences in paired t-test: $p < 0.01$ (†).

Because injection of alanine caused an increase in proline concentration similar to that achieved by AKH peptides (Fig. 35), some other free amino acids were injected into *P. sinuata* (Table 13). No free amino acid other than alanine, however, could significantly elevate the haemolymph proline concentration. The increase of proline concentration after alanine injection was dose-dependent (Fig. 39).

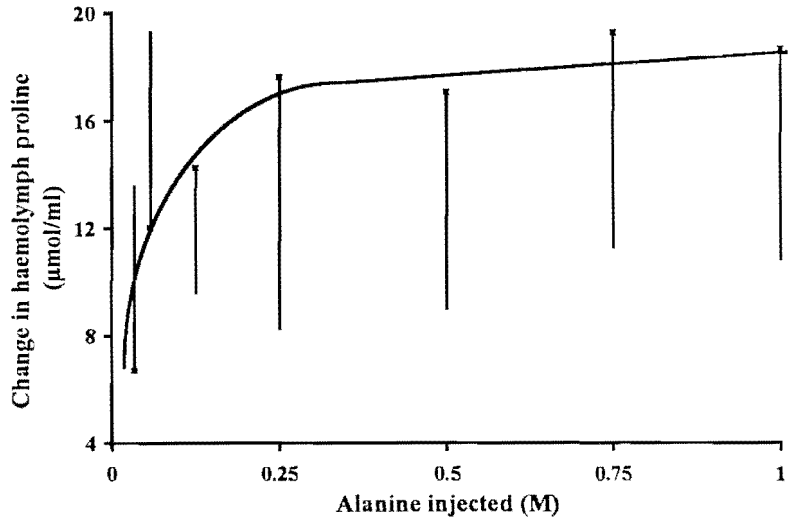

Figure 39. Dose - response relationship of the influence of injected alanine on haemolymph proline concentration of *P. sinuata*. Values are means ± S.D. (n = 12 - 13).

3.6.3 AKH peptides in corpora cardiaca of various scarab beetles

3.6.3.1. Biological activity

Corpora cardiaca extracts of a number of cetoniine and melolonthine scarab beetles were run on HPLC and the UV and fluorescence traces were compared with those of *P. sinuata* (fluorescence traces depicted in Fig. 40) and *T. fascicularis* (fluorescence traces depicted in Fig. 41), which both contain Mem-CC (Gäde *et al.*, 1992). All extracts tested contained a peptide which eluted at the same retention time as Mem-CC. The corpora cardiaca of *P. sinuata* contained a second peak, which was shown to have hyperprolinaemic activity [see Fig. 32 (fraction 11), Fig. 33 and Table 8] in conspecific bioassays. Two *Dischista* species and *Leucocelis amethystina* contained peptides which are most likely identical to the novel *P. sinuata* peptide, because they eluted at the same retention time as the latter (Fig. 40). Peak material of *P. sinuata* was available in sufficient amounts and its biological activity was therefore further investigated. In the conspecific bioassay a small hyperglycaemic effect could be demonstrated (Table 14). The concentration of total carbohydrates rose from 7.0 ± 3.4 to 11.4 ± 4.8 mg/ml upon injection of one gland equivalent, while the concentration increased only by 0.2 ± 1.4 mg/ml from 5.2 ± 3.9 to 5.4 ±3.4 mg/ml in the control group (injection of distilled water).

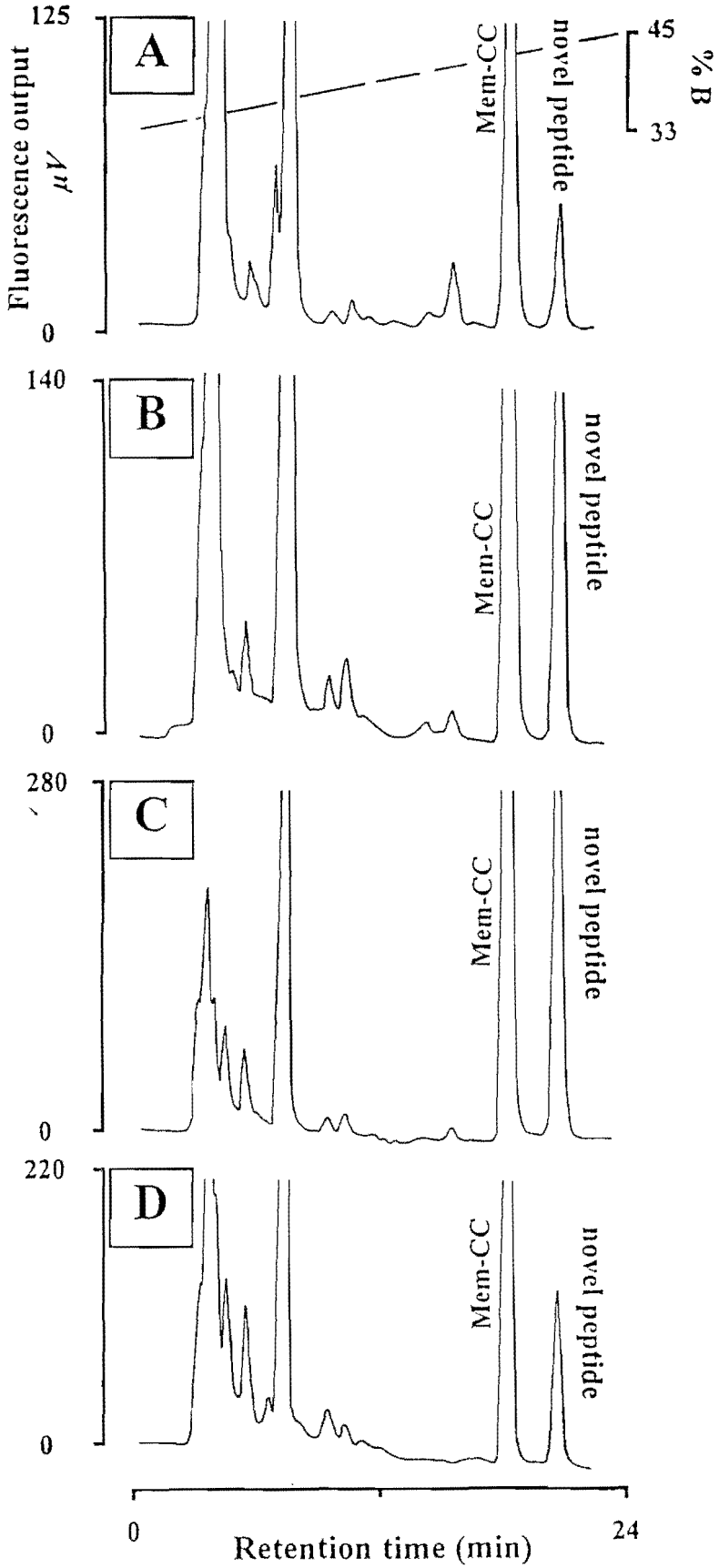

Figure 40. Comparison of corpora cardiaca material of 3 beetles species with that of *P. sinuata*. Fluorescence signal from methanolic gland extracts of: A. *P. sinuata* (15 glands), B. *Dischista cincta* (10 glands), C. *D. rufa* (8 glands) and D. *Leucocelis amethystina* (12 glands).

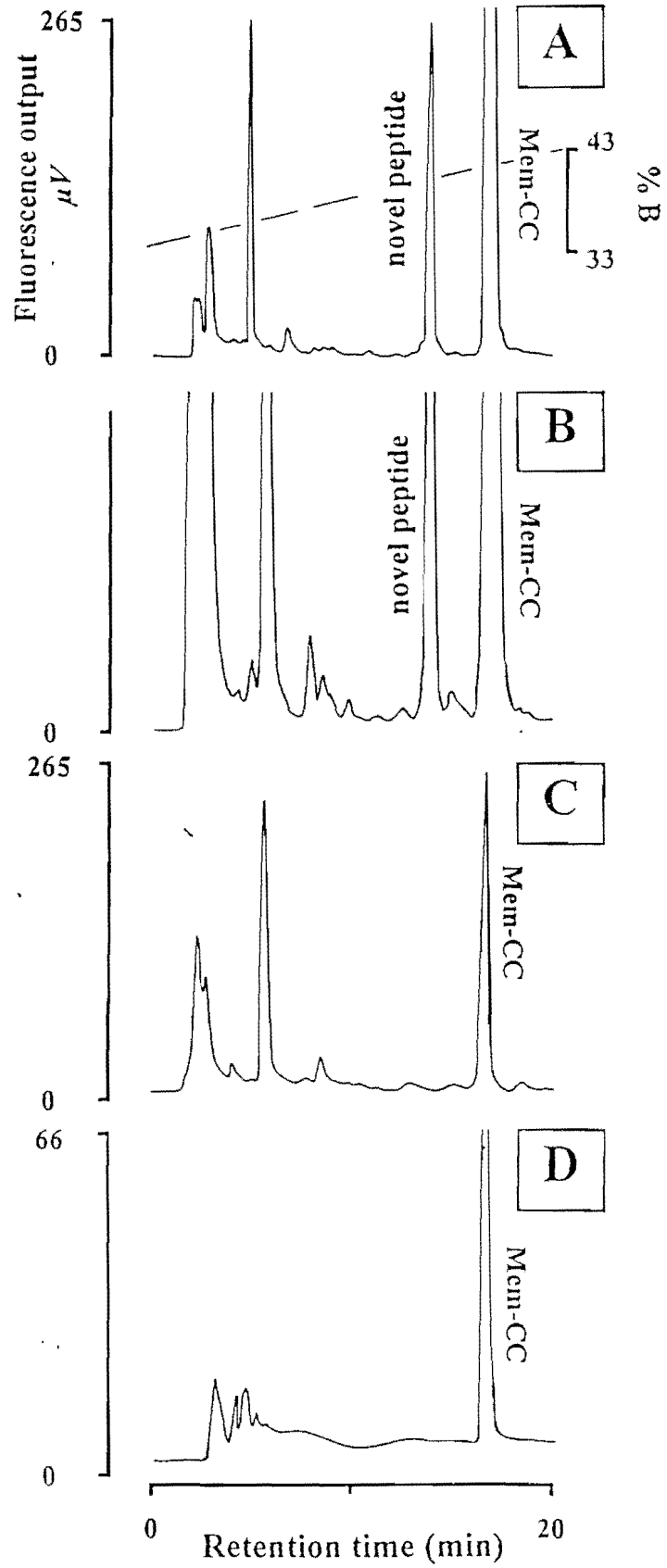

Figure 41. Isolation of neuropeptides from several beetles. Fluorescence signal from methanolic gland extracts of: A. *Trichostetha fascicularis* (10 glands), B. *T. albopicta* (8 glands), C. *Lepithrix* sp. (20 glands) and D. *Camenta innocua* (20 glands).

Table 14. Adipokinetic, hypertrehalosaemic and hyperprolinaemic activity of HPLC peak material of corpora cardiaca extracts of *P. sinuata* and *T. fascicularis*.

	P. sinuata				*L. migratoria*	*P. americana*	*Trichostetha fascicularis*			
	change in haemolymph				change in haemolymph	change in haemolymph	change in haemolymph			
	proline (µmol/ml)	**alanine** (µmol/ml)	**lipids** (mg/ml)	**carbohydrates** (mg/ml)	**lipids** (mg/ml)	**carbohydrates** (mg/ml)	**proline** (µmol/ml)	**alanine** (µmol/ml)	**carbohydrates** (mg/ml)	**lipids** (mg/ml)
treatment	n		n	n	n	n	n			
Control (distilled water)	6 -3.0 ± 6.2	-2.6 ± 5.2	7 -0.1 ± 2.1	7 0.2 ± 1.4	4 -2.7 ± 4.8	5 1.6 ± 2.3	6 1.9 ± 10.3	-0.1 ± 0.8	-3.4 ± 3.5	-1.1 † ± 0.9
Mem - CC (50 pmol)	6 30.2 †† ± 14.9	-8.4 †† ± 3.7	7 0.5 ± 1.8	7 13.7 † ± 6.1			5 34.8 †† ± 9.7	-2.9 †† ± 1.1	3.0 † ± 2.9	-0.7 ± 1.0
Locust extract[1]					6 38.8 † ± 23.2					
Cockroach extract[1]						6 18.5 †† ± 7.5				
peptide I[2,3]	10 16.2 †† ±4.1	-5.2 †† 2.0	10 0.1 ± 1.1	10 4.4 †† ± 2.8	8 2.8 † ± 1.9	8 5.1 †† ± 2.5				
peptide II[2,4]	10 12.6 †† ± 9.1	-9.0 †† ± 4.2			9 0.8 ± 3.8	8 0.7 ± 4.7	10 37.4 †† ± 22.7	-2.4 †† ± 2.0	4.5 †† ± 2.8	-0.4 ± 0.8

Values are given as means ± S.D.; [1] 0.1 gland equivalent, [2] 1 gland equivalent. [3]Novel peptide of *P. sinuata* as depicted in Fig. 40A. [4]Novel peptide of *T. fascicularis* as depicted in Fig. 41A. Significance levels of differences between value before and after injection: † $p < 0.05$, †† $p < 0.002$.

The peak material also caused a significant increase of haemolymph proline concentration from 98.1 ± 9.2 to 114.3 ± 11.7 µmol/ml and a concomitant decrease of haemolymph alanine concentration from 7.2 ± 4.1 to 2.0 ± 1.3 µmol/ml in the conspecific bioassay (Table 14).

The purified gland material had, however, no effect on the haemolymph lipid concentration of *P. sinuata*. The pre-injection value of 7.1 ± 2.9 mg/ml was virtually unchanged with 7.2 ± 4.1 mg/ml after injection. No significant lipid changes occurred after injection of distilled water or 50 pmol of Mem-CC.

When the peak material was used in a heterospecific bioassay with *L. migratoria* as an acceptor, haemolymph lipid concentration increased by 2.8 mg/ml (from 8.8 ± 2.3 to 11.6 ± 2.1 mg/ml; Table 14). The injection of 0.1 equivalent of locust gland material as a 'positive control' caused an increase of the haemolymph lipids from 9.5 ± 4.7 to 48.3 ± 24.6 mg/ml.

When peak material of one gland equivalent was injected into *P. americana*, only a slight, but significant increase of the total haemolymph carbohydrates occurred from 11.6 ± 0.9 to 16.7 ± 3.0 mg/ml, while the injection of its own crude extract at 0.1 cockroach gland equivalent resulted in an elevation of the carbohydrate levels from 13.6 ± 2.7 to 32.1 ± 8.3 mg/ml (Table 14).

The gland material of the melolonthine beetles (*C. innocua* and *Lepithrix* sp.) (Fig. 41C, D) which were compared with *T. fascicularis*, contained a peptide that would most likely be Mem-CC, because it eluted at the same retention time as the Mem-CC of *T. fascicularis* glands. Sufficient material was available from *C. innocua* for deblocking and sequencing this peptide (see below).

In addition to the predominant Mem-CC peak in corpora cardiaca extracts of *T. fascicularis* and *T. albopicta* that were run on HPLC, a much smaller UV and fluorescence peak eluted earlier than Mem-CC (Fig 41A, B). This peak did not occur under the same HPLC conditions in any of the other beetles tested (Fig. 40 and 41). Material of this peak of *T. fascicularis* was purified in sufficient amounts to investigate biological activity and to obtain some structural information.

In conspecific bioassays, the peak material was shown to have hyperprolinaemic activity and, additionally, had a small hyperglycaemic effect (Table 14). The haemolymph proline concentration rose from 98.4 ± 34.9 to 135.8 ± 27.5 µmol/ml upon injection of 1 gland equivalent of peak material, while the alanine concentration dropped from 2.7 ±

2.3 to 0.3 ± 0.7 µmol/ml (Table 14). Total haemolymph carbohydrate concentration rose from 12.4 ± 4.2 to 16.9 ± 5.9 mg/ml, while injection of distilled water did not lead to an increase (Table 14). Injection of 50 pmol Mem-CC as a 'positive control' resulted in a rise of the carbohydrate levels from 18.8 ± 5.1 to 21.8 ± 6.3 mg/ml. Lipid concentration of *T. fascicularis* was neither affected by the injection of peak material nor by the injection of Mem-CC, but slightly by injection of distilled water (Table 14).

While the peak material had no hyperlipaemic activity when injected in locusts and no hyperglycaemic activity in cockroaches (for control values see above), the substance did cause hyperprolinaemia in *P. sinuata*. The haemolymph proline concentration rose from 99.3 ± 15.5 to 112.4 ± 15.3 µmol/ml and alanine concentration dropped from 11.5 ± 3.5 to 2.5 ± 3.1 µmol/ml after injection of one *T. fascicularis* gland equivalent of peak material (Table 14).

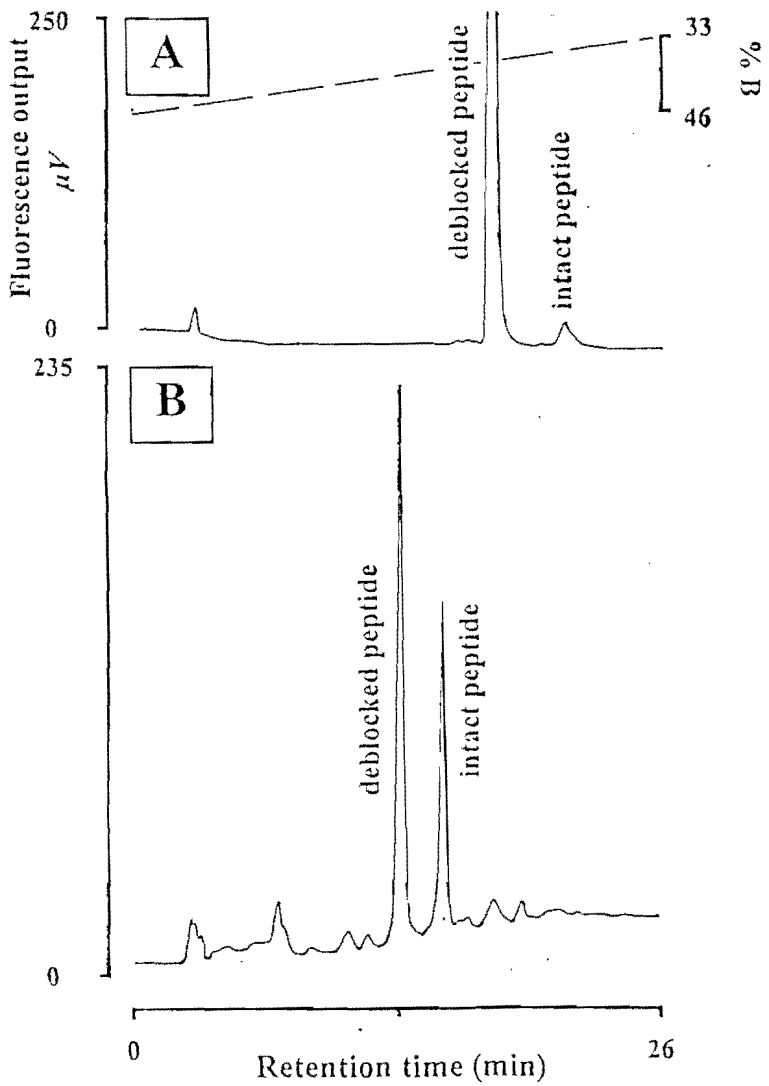

Figure 42. HPLC fluorescence trace of novel peptides found in corpora cardiaca of A. *P. sinuata* (see Figs. 32, 33 and 40A) and B. *T. fascicularis* (see Fig. 41 A) after digestion with pyroglutamate aminopeptidase. The deblocked peptides elute earlier than the intact peptides, respectively.

3.6.3.2. Structural information

The three peptides selected for structural investigation gave a fluorescence signal, indicating the presence of a tryptophane residue. In Figure 42 fluorescence traces of HPLC runs of the novel peptides from *P. sinuata* (A) and *T. fascicularis* (B) are shown after they were digested with pyroglutamate aminopeptidase. In both cases, a new peak appeared which eluted several minutes earlier than the original intact peptide. The same was true for the *C. innocua* peptide (not shown). This indicated that all three peaks were peptides with pyroglutamate residues at their N-terminals.

After removal of the pyroglutamate residue by pyroglutamate aminopeptidase, it was possible to analyse the primary structure of the peptides by automated Edman degradation (Table 15). The residues determined were:

peptide of *P. sinuata*: (pGlu) - Ile - Asn - Leu - Thr - **XAA** - Gly - Trp

peptide of *T. fascicularis*: (pGlu) - Ile - Asn - Met - Thr - **XAA** - Gly - Trp

peptide of *C. innocua*: (pGlu) - Leu - Asn - Tyr - Ser - Pro - Asp - Trp

Position 6 (**XAA**) of the peptides of *P. sinuata* and *T. fascicularis* caused a gap during sequencing. The sequence of the *C. innocua* peptide is identical with Mem-CC. Mass spectrometry with the new peptides of *P. sinuata* and *T. fascicularis* gave no clear result.

Table 15. Amino acid sequences of peptides purified from corpora cardiaca of *P. sinuata*, *T. fascicularis* and *C. innocua*.

	Peptide recovery (pmol) from				
	P. sinuata		*T. fascicularis*		*C. innocua*
Amino acid		Amino acid		Amino acid	
Ile[1]	75.4	Ile[1]	96.9	Leu[1]	25.7
Asn[2]	34.2	Asn[2]	72.1	Asn[2]	15.9
Leu[3]	70.7	Met[3]	74.3	Tyr[3]	15.5
Thr[4]	37.1	Thr[4]	20.5	Ser[4]	3.0
XAA[5]	-	**XAA**[5]	-	Pro[5]	6.1
Gly[6]	33.3	Gly[6]	37.2	Asp[6]	6.6
Trp[7]	3.3	Trp[7]	12.0	Trp[7]	2.0
-[8]	-	-[8]	-	-[8]	-

The N - terminal pyroglutamyl residue of the HPLC - purified peptides was cleaved off enzymatically, and the deblocked peptides were analysed.

3.7. Proline synthesis in the fat body of *P. sinuata*

3.7.1. The place of proline re-synthesis

An experiment was conducted to locate the tissue where proline synthesis in *P. sinuata* takes place. Different tissues of the abdomen and flight muscle tissue were incubated in saline containing alanine (20 mM) and proline production was measured (Table 16). Fat body tissue displayed the highest rate of proline synthesis, while flight muscle tissue released only small amount of proline which was negligible when compared with the rate of fat body tissue. Gut tissue and spermatophores did not produce proline under the same conditions. (Table 16).

Table 16. Proline synthesis of different tissues of *P. sinuata* in a medium containing alanine (20 mM).

Tissue	Proline production (µmol/h x mg protein)
fat body	2.32 ± 0.27
gut	0.09 ± 0.09
spermatophors	0.02 ± 0.01
flight muscles	0.21 ± 0.08

Values are given as means ± S.D., n = 5.

3.7.2. Precursors for proline synthesis

When fat body tissue of *P. sinuata* was incubated only in *Pachnoda* saline (without alanine), it became clear that proline synthesis took place using endogenous substrates as precursor. Fat body tissue could generate proline for several hours but after 4 hours the rate stabilised at a low level (Fig. 43). Therefore, for all further experiments, the tissue was pre-incubated for 2 x 2 h (see section 2.9.2.).

Results in section 3.8.1. had shown that alanine is a precursor for proline synthesis. This was confirmed when fat body tissue was incubated in a medium containing unlabelled alanine (20 mM) plus 3 µCi of radioactive label in the form of ^{14}C-UL-alanine. The radiocarbon which was located in the alanine in the medium before incubation (Fig. 44A), was incorporated into proline during the time course of incubation (Fig. 44B) and after 150 min of incubation was found only in the proline released from the fat body into the medium (Fig. 44C).

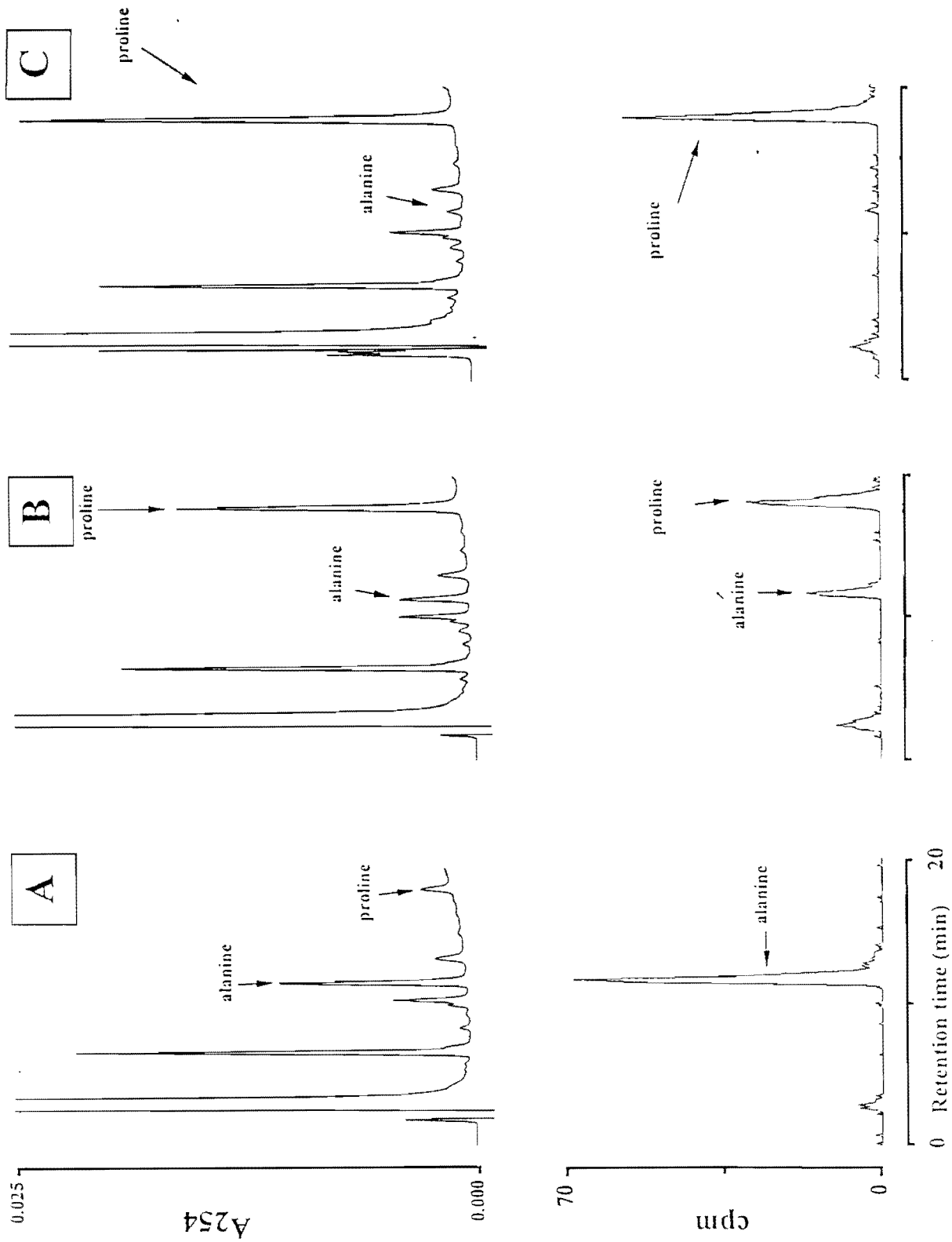

Figure 44. Incorporation of radiocarbon from ^{14}C-UL-labelled alanine into proline in the fat body of *P. sinuata in vitro*. The medium contained also non radioactive alanine (20 mM). The figure shows UV traces (top) and β - radiation trace (bottom) of samples taken from the incubation medium before (A) and after 60 (B) and 150 min of incubation of fat body tissue (of one beetle) and run on HPLC after derivatisation as described in Materials and Methods.

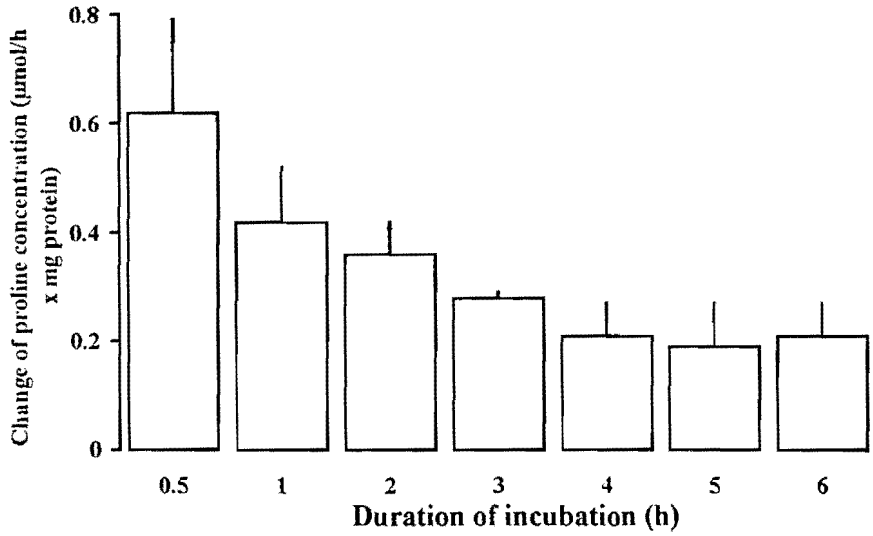

Figure 43. Proline synthesis from endogenous substrates is depicted during incubation of *P. simuata* fat body tissue (n = 5) in *Pachnoda* saline at 35 °C. Samples were rinsed and transferred into new medium after each incubation period. Samples for amino acid determination were taken before and after each incubation period and the change of proline concentration calculated.

Several unlabelled amino acids and glucose were subsequently tested for their potential to stimulate proline synthesis (Table 17). Glutamate and aspartate led to a similar proline release into the incubation medium as with alanine, but no proline production occurred when leucine and glucose were tested (Table 17).

Table 17. Proline synthesis of fat body of *P. simuata*

	addition of substrate (10 mM):				
	Ala	**Asp**	**Glu**	**Leu**	**Glucose**
Difference of proline synthesis in comparison to the respective left half of fat body (µmol/h x mg protein)					
mean	2.88	2.61	2.67	-0.01	-0.03
± SD	0.18	0.27	0.34	0.08	0.05

Values are expressed as means ± S.D., n = 6.

In Table 18 results of incubation experiments are presented, where radioactively labelled compounds were tested for their capability to supply carbon units for proline synthesis. Incorporation of radiocarbon was highest, when ^{14}C-UL-alanine or ^{14}C-UL-aspartate plus unlabelled alanine (20 mM) were present in the medium, while no radiocarbon of ^{14}C-UL-tyrosine and only small amounts from ^{14}C-UL-palmitate was introduced into proline (Table 18). When the latter compounds were applied in the

presence of cold alanine (20 mM), radiocarbon from ^{14}C-UL-palmitate was observed in proline, while that of ^{14}C-UL-tyrosine was not.

Table 18. *In vitro* incorporation of radiocarbon from ^{14}C - labeled substrates into proline in the fat body of *P. sinuata*.

labeled compound	n	proline synthesised (µmol/h x mg protein)	radioactivity incorporated (cpm/h x mg protein)
alanine	5	3.21 ± 0.12	759893 ± 98502
aspartate[1]	3	2.71 ± 0.26	829943 ± 55361
aspartate	3	0.18 ± 0.02	94432 ± 2056
tyrosine[1]	3	3.03 ± 0.18	0
tyrosine	3	0.25 ± 0.11	0
palmitate[1]	4	2.97 ± 0.36	79983 ± 14223
palmitate	4	0.12 ± 0.05	5322 ± 427

Data are presented as means ± S.D.

[1]Incubation medium contained alanine (20 mM).

3.8.3. Feedback inhibition of proline synthesis

From *in vivo* biotests with AKH peptides and injection of alanine, it was known that proline haemolymph concentration never exceeded a level of around 120 µmol/ml (see 3.7.1.). Therefore, it was concluded that proline inhibited its own synthesis by feedback. To prove this hypothesis, the *in vitro* alanine consumption of fat body tissue was used as a measure for proline production when different initial amounts of proline were added to the medium (Table 19). With increasing concentration of proline in the medium, alanine consumption (thus proline synthesis) was suppressed from 2.42 µmol/h/mg protein with no addition of proline to 0.53 µmol/h/mg protein with 100 mM proline in the medium. When the medium contains proline at an initial concentration of 100 mM which was found in the haemolymph of resting *P. sinuata* (see 3.1.1.), alanine consumption is only 22 % of that without addition of proline.

Table 19. Influence of proline concentration in the incubation medium on the rate of proline synthesis (measured as alanine consumption).

Initial proline content of the medium (mM)	Consumption of alanine (µmol/h x mg protein)	n
0	2.42 ± 0.31	4
5	2.36 ± 0.25	4
10	2.18 ± 0.18	3
25	1.47 ± 0.09*	5
50	0.82 ± 0.05	4
75	0.6 ± 0.06	4
100	0.53 ± 0.08	5

values are presented as means ± S.D.
*First concentration which inhibited alanine consumption significantly ($p < 0.001$, compared with incubation without proline).

3.8.4. Lipids as a source of acetyl-CoA for proline re-synthesis

When pre-incubated fat body tissue was transferred into a medium containing alanine (20 mM), it was observed that the release of proline from the fat body tissue into the medium is equimolar to the disappearance of alanine (Table 20). This raises the question of where the additional two carbons come from. Fat body glycogen and lipids are the major sources which can supply these C_2 units via acetyl-CoA to the Krebs cycle. *In vitro* fat body incubation with glucose (Table 17) did not lead to a substantial proline production. Aspartate and glutamate caused *in vitro* proline production comparable to alanine (Table 17) and radiolabelled aspartate was shown to be incorporated into proline (Table 18). Injection of both amino acids, however, did not elevate haemolymph proline levels *in vivo* (Table 13). Additionally, they are not available in amounts comparable to alanine during and after flight. Therefore, research was focused on the lipid metabolism as the possible source of acetyl-CoA for proline synthesis.

Table 20. Relation between alanine utilisation and proline synthesis in the fat body of *P. sinuata* in vitro.

	Change of concentration in medium (µmol/h x mg protein)		
Alanine in medium (mM)	Alanine	Proline	Difference of change
20	-2.15 ± 0.30	2.18 ± 0.29	0.03 ± 0.26
0*	-	0.08 ± 0.06	

*Medium contained only *Pachnoda* saline without additions.
Values are means ± S.D., n = 12.

Gas chromatography of the lipids of *Pachnoda* fat body tissue showed that oleic acid is the predominant fatty acid of the lipids with 59 % and palmitic acid accounted for 30 % (Table 21). Despite the predominance of oleic acid it was decided to use palmitic acid for experimentation because it was also present at a high percentage. In addition, it was commercially available in the universally labelled form, while carbon labelled oleic acid could have been bought only in the ^{14}C-1-form. The fact that radiocarbon from ^{14}C-UL-palmitate was incorporated into proline (see Table 18) confirmed this decision.

Table 21. Relative fatty acid content of *P. sinuata* fat body lipids

Fatty acid		% of total fatty acids
myristic acid	C 14 : 0	1.0
palmitic acid	C 16 : 0	30.0
palmitoleic acid	C 16 : 1	2.8
stearic acid	C 18 : 0	4.9
oleic acid	C 18 : 1	59.0
linoleic acid	C 18 : 2	1.6
arachidic acid	C 20 : 0	0.7

Sample contained fat body tissue of 10 animals.

An experiment was conducted to examine whether the ^{14}C-UL-palmitate was incorporated into the fat body cells (Table 22). As already shown in Table 18, little radiocarbon could be observed in synthesised proline, when ^{14}C-palmitate was applied without alanine. After 2 h of incubation with ^{14}C-UL-palmitate the tissue was washed with saline and each sample was split into three sub-samples. Sub-sample one was examined immediately (see below), the second sub-sample was transferred into new medium containing only cold alanine (20 mM), and the third sub-sample was transferred into *Pachnoda* saline without additions. In sub-sample two, where alanine was present in the medium, radiocarbon was observed in proline, while in the control sample (sub-sample three) hardly any label was hardly incorporated (Table 22).

Sub-sample one was examined in order to find out into which metabolites the radiolabel was incorporated. After extraction of fat body tissue (see 2.9.3.) radioactivity of the lipid- and non-lipid phase were measured. The majority of the radiolabel, 98.4 ± 8.8 % (303721 ± 12237 cpm/mg protein) was present in the lipid phase, while it was negligible with 1.6 ± 0.4 % (4992 ± 1298 cpm/mg protein) in the non-lipid phase (n = 3).

Material of the lipid phase was subsequently subjected to thin layer chromatography (TLC). Results showed that the majority of the radiocarbon (87.8 ± 7.7

%) was incorporated into the triglyceride fraction and was present to a smaller extent in the free fatty acid fraction (1.4 ± 0.7 %) and various other fractions (Fig. 45).

Table 22. *In vitro* incorporation of radiocarbon from ^{14}C - palmitate into *Pachnoda sinuata* fat body and its release with alanine as precursor.

radiocarbon incorporated into proline (cpm/hxmg protein)	
sample incubated in saline[1]	
6711 ± 478	
sub - samples incubated in saline	sub - samples incubated in saline + alanine (20 mM)
1067 ± 223	56545 ± 1892

Values are means ± S.D., n = 3.

[1]Tissue samples were washed, divided into two sub - samples and transferred into new medium.

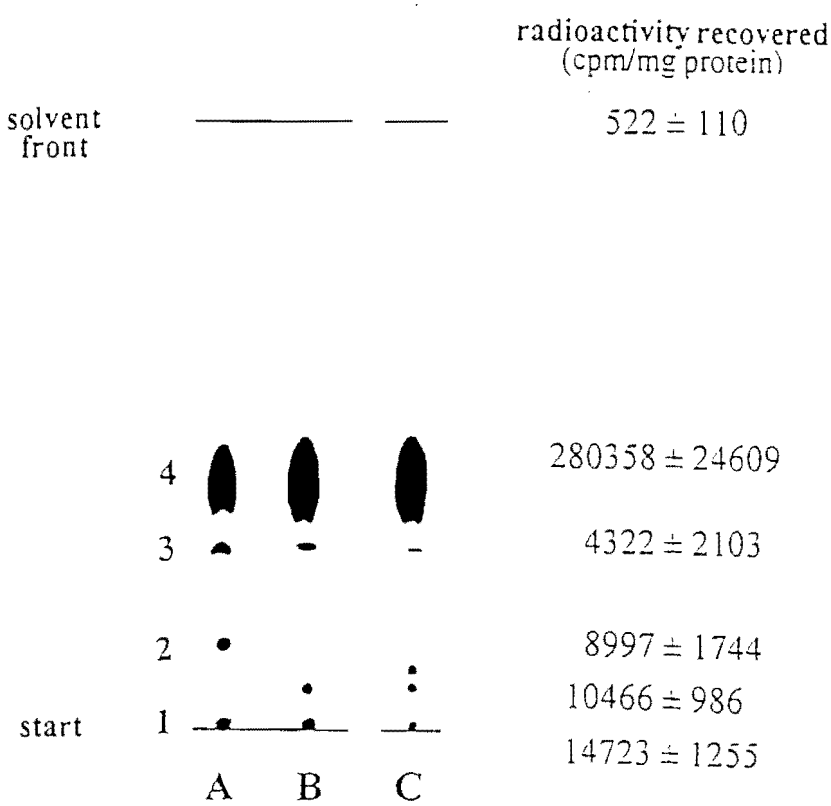

Figure 45. Incorporation of radiocarbon from ^{14}C-UL-palmitate into various lipid fractions of *P. sinuata* fat body as separated by TLC. The drawing was made from representative runs of a standard mixture (A), containing (1) monoolein, (2) diolein, (3) palmitate + oleate and (4) triolein, and lipids extracted from *P. sinuata* fat body (B). (C) shows the autoradiogram of (B). The radioactivity recovered from the various fractions is given on the right side of (C) as means ± S.D. (n = 3).

4. DISCUSSION

4.1. Energy substrates for flight in *Pachnoda sinuata*

4.1.1. Concentration of flight substrates in the haemolymph

In the open circulatory system of insects the haemolymph links the flight muscles, where the oxidation of energy substrates takes place, with the fat body, the most important storage site in insects and a place of various syntheses. The function of the fat body is, in many respects, comparable with that of vertebrate liver and adipose tissue. The reserves in the flight muscles are very limited and last only for the early phase of flight. In *Locusta migratoria,* for example, flight muscle glycogen is used only during the first 3 min of tethered flight (Worm and Beenakkers, 1980) while in *Schistocerca gregaria* two thirds of the flight muscle glycogen stores are depleted in only 10 s of tethered flight (Rowan and Newsholme, 1979). Goldsworthy (1983) assumed that these stores in locust flight muscles are only sufficient to power 2-3 min of trivial flight. Therefore, substrates have to be released from the fat body and transported via the haemolymph to the flight muscles for use during migratory flight (Goldsworthy, 1983). Besides this transport function, the haemolymph is an important storage site for substrates itself. For instance, in *L. migratoria* the haemolymph pool is the exclusive source of trehalose during the first 30 min of tethered flight (van der Horst *et al.,* 1978).

To fulfil this supply function shortly after take-off, the concentration of the initial fuel(s) in the haemolymph must be high. Resting levels of metabolites in the haemolymph can therefore give some information about the potential fuels in an insect. This is especially true for the fuels which are used shortly after the onset of flight and which have to be available immediately. Locusts, which initially use carbohydrates before they switch to lipids, have resting carbohydrate levels as high as 32 mg/ml (Jutsum and Goldsworthy, 1976) in their haemolymph and in cicadas the level is even higher with about 60 mg/ml (Gäde and Janssens, 1994). Very high lipid concentrations have been reported in the haemolymph of the moth, *Imbrasia cynthia* (Liebrich and Gäde, 1995). In the present study it was shown that in the haemolymph of *P. sinuata,* proline dominated with resting levels of around 90-100 µmol/ml, while there were only moderate levels of carbohydrates, with 10-12 mg/ml. High resting concentrations of proline were also found in the haemolymph of other scarab beetles investigated. In *D. lunata* both proline and

carbohydrate levels were high, while in the carpenter bee, *Xylocopa capitata*, haemolymph carbohydrate levels were the dominant fuel.

From the haemolymph data it was initially thought that proline was a major fuel in most of the beetles investigated, while *X. capitata* predominantly used carbohydrates for flight. The data from *D. lunata* suggest that in this beetle, both carbohydrates and proline play an important role as energy substrates during flight. Resting levels of haemolymph proline higher than those recorded in *P. sinuata* have only been found in the tsetse fly, where levels reach up to 174 µmol/ml (Bursell, 1963).

The resting concentration of carbohydrates in the haemolymph of *P. sinuata* is less than that in the locust, suggesting that it is less important than proline for the initial phase of flight, or for flight preparation.

In *L. migratoria* an interaction of carbohydrates and lipids during their oxidation in the flight muscles was shown with high concentrations of diglycerides suppressing the consumption of trehalose (Robinson and Goldsworthy, 1976, 1977). On the other hand, the effect could be reversed by injection of trehalose, indicating a competitive inhibition (Robinson and Goldsworthy, 1976). The oxidation rate, therefore depends on the relative substrate concentrations (in the absence of AKH peptides). It was also shown that the rate of oxidation depends on the concentration of the particular substrate. In the haemolymph of *P. sinuata* the proline concentration is very high, indicating that it is not only an important flight substrate but most likely serves as an initial fuel as well. It was demonstrated experimentally that the proline concentration in the haemolymph and the flight muscles indeed dropped as soon as the beetles took off (see 3.1.1.). In addition, it was shown that proline was already in use during flight preparation of *P. sinuata* (discussed in chapter 4.2.1.). It may well be that the oxidation of carbohydrates is inhibited by the high proline concentrations present at the onset of flight, which is only relieved when the proline level drops during the course of flight. Such a hypothesis is supported by interactions of proline and pyruvate at the mitochondrial level, which are discussed in section 4.3.2.

4.1.2. Substrates for respiration of flight muscle mitochondria

Some information about the enzymatic design of the flight muscles, and thus indication for the potential fuels for flight, was obtained by the measurement of respiration rates of flight muscle mitochondria of *P. sinuata*. These data were compared

with those measured with flight muscle mitochondria from *L. migratoria* and *D. lunata*. Proline was the substrate which led to the highest oxidation rates (see 3.5.) in *P. sinuata*. The respiration rate was also very high when pyruvate was added to the incubation medium. Similar results were obtained when flight muscle mitochondria of the blister beetle were used, whereas those of locust flight muscles oxidised not only proline, but also pyruvate, at much lower rates than preparations of flight muscle mitochondria from fruit beetles (Table 7). This is to be expected, at least for proline, which is only of minor importance for locust flight (Worm and Beenakkers, 1980). In the other three scarab beetles investigated (*T. fascicularis, Lepithrix* sp., *C. innocua*), respiration of flight muscle mitochondria with proline and pyruvate as substrates was comparable with that in *P. sinuata* (Table 7). This provided evidence that these three beetle species apparently have a similar flight metabolism to the fruit beetle. Compared with the data reported by Weeda *et al.* (1980a) for flight muscle mitochondria of the Colorado potato beetle, respiration rates for all beetles (except *D. lunata*) were approximately three-fold higher, at 3 to 4 µg-atoms O_2/min x mg protein measured with proline as substrate, and about ten-fold higher, at about 3 µg-atoms O_2/min x mg protein with pyruvate, in the present study. This may be due to different experimental conditions rather than a physiological difference, because the Colorado potato beetle is known to use carbohydrates and proline to power its flight (Weeda *et al.*, 1979). The high rates of proline oxidation obtained with the beetles species in the present study are in the same high range as those found in experiments with flight muscle mitochondria of the tsetse fly, an exclusive proline user (Slack and Bursell, 1976). The rates of pyruvate respiration in the present work were in the range of previous data recorded for pyruvate oxidation in flight muscle mitochondria of the blowfly, a carbohydrate user (Slack and Bursell, 1976).

During glycolysis, glycerophosphate is formed in equimolar amounts compared with pyruvate and it is part of the glycerophosphate cycle in insects which re-oxidises NADH formed glycolytically in the cytosol. Glycerophosphate was oxidised at a high rate by *P. sinuata* flight muscle mitochondria, similar to those of the blister beetle and the other beetles tested (Table 7). The respiration rate of locust mitochondria, however, is much higher. In all the insects tested, a very active glycerophosphate cycle was present, confirming the importance of the breakdown of carbohydrates during flight in those insects. In flight muscle mitochondria of all test insects, respiration rate with glycerophosphate could be increased by addition of Ca^{2+}. This is most likely by direct

action on the mitochondrial α-glycerol-phosphate dehydrogenase which is activated by divalent ions (Chance and Sacktor, 1958; Hansford and Chappell, 1967).

Other amino acids tested (glutamate, aspartate, alanine) resulted in much lower state 3 respiration in all species examined. It is most likely that this is because the inner mitochondrial membrane is impermeable to these amino acids (Sacktor and Childress, 1967).

The same is true for Krebs cycle intermediates (citrate, oxoglutarate, malate) which gave low respiration rates when flight muscle mitochondria of several beetle species were determined. This is an indication that the mitochondria used in this study were mostly intact, because these compounds are not able to cross the inner membrane of undamaged mitochondria (Chappell and Crofts, 1966). However, it was not possible to demonstrate a substantial respiration rate of *P. sinuata* flight muscle mitochondria with palmitoyl-carnitine, while preparations of locust flight muscles oxidised the substrate at a high rate (Table 7). This makes it unlikely that lipids can be oxidised by *P. sinuata* flight muscles during flight.

In addition to the poor capability of the different flight muscle mitochondria preparations to oxidise exogenous amino acids (except proline) and Krebs cycle intermediates (see above), other results indicate that the preparations used in the present study were of high quality. In contrast to the work of Zebe and Gäde (1993), it was not necessary to add malate as a primer for pyruvate oxidation, because intact mitochondria did not lose internal substrates during the isolation process. The more gentle "Nagarse" method is therefore the method of choice rather than the use of a Potter-Elvejem homogeniser. Another measure for the intactness of the isolated mitochondria was the high respiratory control ratio (RCR) for the two substrates calculated for *P. sinuata* flight muscle mitochondria (Table 7). This suggests that oxidation of the substrates was tightly coupled with phosphorylation. RCR describes the ratio of the respiration rate in the presence of ADP (state 3 respiration) and the resting rate (state 4 respiration) when no ADP is available (Estabrook, 1967). In addition, the ADP:O ratio (P/O), which was calculated from the traces of state 3 respiration according to Estabrook (1967), was approaching the theoretical limit of 3 for NAD-dependent substrates.

4.1.3. Maximum enzyme activities in the flight muscles

To determine the metabolic flux of a certain pathway, it is necessary to measure the activity of the key enzymes which catalyse the non-equilibrium reactions of the pathway and, therefore, regulate the pace (Newsholme and Crabtree, 1986). However, to estimate the significance of a pathway, maximum activities of near-equilibrium enzymes can be measured. Such near-equilibrium enzymes occur in constant proportions to the other enzymes of the same pathway (according to Pette, reviewed in Beenakkers *et al.*, 1984). Thus, measurement of a single enzyme of a pathway can be applied to describe its importance. The latter method was used in *P. sinuata* to estimate the importance of the possible flight substrates. The values were compared with those of flight muscles from locusts and blister beetles.

High activities of GluDH and AlaT were measured in *P. sinuata* as well as *D. lunata* flight muscles (Table 5). Both enzymes are capable of introducing carbon moieties of glutamate, the intermediate of proline catabolism, into the Krebs cycle. These results confirm results reported by Zebe and Gäde (1993) for the fruit beetle and, in addition, that proline is an important flight substrate in the blister beetle.

The high activity of AlaT demonstrates the presence of an efficient transamination system, a pre-requisite of partial proline oxidation, in the two beetles. It is not clear, however, what function the GluDH has, due to the roughly equimolar changes of proline and alanine concentrations during flight in the two beetles (see chapters 3.1.1. and 3.1.4.). Furthermore, the equimolar proline consumption and alanine production in isolated flight muscle mitochondria of fruit beetles (see chapter 3.5.) suggests that GluDH does not make a major contribution to proline breakdown. Such a contribution would result in a deficit of alanine production in comparison with proline consumption, which was not found in the experiments presented here.

In the locust, both enzymes have very low activities which is in agreement with findings that proline is oxidised only in small quantities during locust flight (Kirsten *et al.*, 1963; Rowan and Newsholme, 1979; Worm and Beenakkers, 1980).

Another enzyme which could introduce carbon skeletons from amino acid breakdown, AspT, had low activity in the fruit beetle and the locust and can, therefore, be excluded as a major contributor of metabolites to the Krebs cycle during flight in either insect.

The activity of the two enzymes which compete for the same substrate at the malate branchpoint of the Krebs cycle, ME and MDH, were measured to draw conclusions about the capability of the flight muscles of each of the respective insects to provide pyruvate as an acceptor for the ammonia, which is released during deamination of glutamate by AlaT. The enzyme responsible for the decarboxylation of malate to pyruvate for subsequent transamination is ME. The ratio of the two enzymes (MDH:ME) is an indicator for the quantitative importance of proline as a fuel for flight in insects (see Bursell, 1981). A low ratio would mean a relatively active ME and a high ratio, a less active ME.

A MDH:ME ratio of 7 was obtained for the blister beetle, which was lower than that of *P. simuata* (MDH:ME = 13), mainly because of the higher MDH activity of the latter. In the locust a ratio of 55 was calculated due to the low ME activity, confirming that this pathway is almost negligible in *L. migratoria*. The ratios obtained for the two beetles species is in the same range as those found in the Colorado beetle (MDH:ME = 8, Weeda *et al.*, 1980a) and the dung beetle, *Heliocarpus* sp. (MDH:ME = 8, Hoek *et al.*, 1976). The Colorado potato beetle was found to use proline in combination with carbohydrates to power its flight (Weeda *et al.*, 1979). The lowest rate (MDH:ME = 1) was discovered in the tsetse fly, *G. morsitans* (Hoek *et al.*, 1976), which oxidises proline as the sole substrate for energy supply during flight (see Bursell, 1981). In the cockroach, however, a MDH:ME ratio of 101 (Hoek *et al.*, 1976) indicates that the flight muscle cells do not have the capacity to decarboxylate enough malate to pyruvate to accept large amounts of ammonia, which results from the proline breakdown. The same can be concluded from the MDH:ME ratio of the locust in the present study.

For the three insects investigated in the present study, the importance of carbohydrate breakdown during flight is underlined by the high activities of GAPDH in the flight muscles. GAPDH is an enzyme of glycolysis and although its activity is much higher than the rates of carbohydrate breakdown *in vivo* (Crabtree and Newsholme, 1975), it is a good measure for the substantial participation of the glycolytic pathway in the substrate supply of the Krebs cycle during flight in the beetles and in the locust (Beenakkers *et al.*, 1984).

The first step in glycogen breakdown is catalysed by glycogen phosphorylase which removes one glucose unit from glycogen and forms glucose-1-P. The activity of

this enzyme measured in locust flight muscles in the present study was high compared with those of other authors, for example the 7.5 U/gfw measured by Crabtree and Newsholme (1972a). Activity for the fruit beetle was threefold higher than those of locusts (Table 5) but were in the range of the activities reported for cockroaches (30 U/gfw) and blowflies (55 U/gfw; Crabtree and Newsholme, 1972a). However, the measured activity of glycogen phosphorylase *in vitro* was too small to account for the observed changes of glycogen concentration in *Pachnoda* flight muscles *in vivo* (Fig. 7B), which were approximately 140 µmol glucose equivalents/min calculated for the first 10 s of lift generating flight of *P. sinuata* (see section 3.1.1.3.). This is probably an indication that the enzyme was not measured under its optimum conditions.

The activation of the enzyme by AMP was found to be 88.5 % in the fruit beetle and 59.8 % in the locust flight muscle.

HOAD is an enzyme of the β-oxidation of fatty acids and, although it does not catalyse the limiting step of the fatty acid breakdown (Crabtree and Newsholme, 1972b), it is still considered to be a good measure of the ability of the flight muscles to directly oxidise lipids (Beenakkers, 1969). The activity of this enzyme found in the fruit beetle is relatively high. It is three times as active as in the blister beetle, but only about a fifth of that of the locust. Schneider *et al.* (1985) could show in the cockchafer, *M. melolontha*, that fat body tissue surrounds the tracheae and is therefore present in the flight muscles. The high activity found in the closely related fruit beetle might therefore be caused by such a "contamination" of flight muscle preparations with fat body tissue, which has a very active HOAD (see Table 6). Although the activity of the HOAD in *P. sinuata* flight muscles was low when compared with that of locust flight muscles, it was in the range of activities measured in a dragonfly species, which was shown to fly predominantly on lipids (Janssens, 1995).

HOAD and GAPDH represent the β-oxidation of lipids and the glycolytic pathway, respectively. The GAPDH:HOAD ratio can therefore be used to estimate the relative importance of fatty acid oxidation during flight (Beenakkers, 1969). The highest GAPDH:HOAD ratio in the present study was observed in the blister beetle (GAPDH:HOAD = 37) followed by the fruit beetle (GAPDH:HOAD = 13). The very low ratio in the locust (GAPDH:HOAD = 2) is an expression of the lipid-based flight

metabolism of this insect. The activity of GAPDH was very similar in flight muscles of *P. sinuata* and *D. lunata*, while the activity of HOAD is three-fold higher in *P. sinuata*. As a result, the GAPDH:HOAD ratio is much lower in the fruit beetle (Table 5).

4.1.4. Patterns of substrate use during flight

In the present study an attempt was made to describe and quantify the pattern of metabolic changes in the African fruit beetle, *P. sinuata*, using a method which simulates flight conditions and which ensured that the beetles had to generate lift (see 2.2.1.). Previous studies with the fruit beetle had only analysed the situation during tethered flight on a flight mill, a method which excluded lift (Zebe and Gäde, 1993; Lopata and Gäde, 1994). Both flight methods were used in an attempt to determine the participation of the different muscles of the flight apparatus in substrate use.

During the time course of flight with and without lift, as well as at rest, the RQ measured in the present study was around 0.9, confirming results that showed that *P. sinuata* uses a combination of proline and carbohydrates to power its flight (Zebe and Gäde, 1993; Lopata and Gäde, 1994; present study). No decrease of RQ was observed during the later stage of flight, which could have indicated the oxidation of fatty acids (Fig. 13).

In lift generating flight two metabolic phases could be distinguished. The first one lasted from take-off until about 30 s of flight. It is characterised by a steep decrease in haemolymph and flight muscle proline and a mirror-image like increase of the alanine concentration, the end product of partial proline degradation (Bursell, 1981; Weeda *et al.*, 1979; see also 4.2.1.). Glycogen concentration in the flight muscle is rapidly diminished, while haemolymph carbohydrate levels increase during this phase. These carbohydrates are probably re-distributed with the haemolymph from other parts of the body after beginning of flight. It is unlikely that they are released from the fat body by the action of the beetle's AKH peptide Mem-CC, because this process was shown to take several minutes (Lopata and Gäde, 1994), which is too long to explain the increase of haemolymph carbohydrates after 30 s as shown here. Flight performance in this early flight period is high, as indicated by the high values of oxygen consumption. It is noteworthy that at the end of this phase, minimum values of the total concentration of proline and alanine occur in the haemolymph (but not in the flight muscles). This

indicates a deficit of Krebs cycle intermediates in early flight, because proline disappears but no comparable amount of alanine arrives in the haemolymph. Thus, a portion of proline might undergo complete oxidation. At this point in the flight pattern, proline re-synthesis in the fat body is not yet generating the maximum amount of proline. Thus, a steady state of proline oxidation and re-synthesis has not been reached yet.

In the second phase of flight, metabolite levels seem to have stabilised. Proline and alanine concentrations in the haemolymph do not change any more, while those in flight muscles do so at a slower rate. It is most likely that the re-synthesis of proline from alanine in the fat body now takes place at a maximum rate, which is at around 1.5 µmol/ml haemolymph x min, as calculated from the first 15 min of rest after flight (Fig. 6A). Glycogen stores in the flight muscle decrease at a much slower rate, while the concentration of carbohydrates in the haemolymph is diminished during this phase. Carbohydrates released from the fat body in the first phase are now available and oxidised, while flight performance is also markedly lower than in the first phase. Thus, the second phase is characterised by the fact that metabolism is in an equilibrium state: lower flight performance results in less demand for fuels and these are provided by using the previously mobilised carbohydrate reserves from the fat body. Because the RQ does not change in this phase compared with the previous one, proline is also contributing to the energy provision.

Because re-synthesis of proline from alanine is taking place in the fat body (see 3.7.), oxidation and synthesis are in keeping with each other and are responsible for the steady state observed in this phase.

The observed two phases during flight seem to be in contrast to the three phases described by Zebe and Gäde (1993). However, these authors studied flight at a flight mill. They found in the first phase, which comprised the first two min of flight, that proline was the exclusive fuel in flight muscles. In the light of the new results of the present study, discussed in 4.3.1., there is a very plausible explanation. Previous studies (Zebe and Gäde, 1993) have not taken into account the pre-flight warm-up phase. It is clear, from metabolic and respiratory studies, that proline is the exclusive fuel during warm-up (3.2.2.); however, this warm-up phase was included in the metabolic changes observed during 2 min of flight in the previous study (Zebe and Gäde, 1993).

All previous flight data for *P. sinuata* were obtained from flight on a flight mill, but such studies investigated only relatively short periods of tethered flight (15 to 20 min;

Barnett *et al.*, 1975; Zebe and Gäde, 1993; Lopata and Gäde, 1994). The flight mill experiments in the present study lasted up to 2 h. Using these data and those of the above mentioned authors, this kind of flight can be characterised as follows: the two phases found during lift generating flight are also obvious in flight without lift, but the metabolic changes occur much slower. The rapid changes discussed for the first phase of lift producing flight (30 s) take place in 4 to 8 min in flight muscles and 8 to 30 min in the haemolymph during flight on the flight mill. The increase of haemolymph carbohydrate levels, which occurred in the first phase of flight with lift production, appeared only after more than 30 min of flight at a flight mill, while at this time fat body glycogen reaches a low and stable level. Most likely, the carbohydrates in the haemolymph derived from fat body reserves. As indicated by measurements of flight velocity, at this stage flight performance reaches a low level.

Beetles stopped flying much later when lift was minimised. While the maximum flight duration of beetles flying with lift was 20 min, some individuals flew for almost three hours on the roundabout. This indicates that the energy expense of flight without lift is much lower than that of lift generating flight. The present results of V_{O_2} measurements in the beetle (see 3.1.2.) confirm this. It is also in accordance with the observations of Heinrich (1971) who measured V_{O_2} of 45-50 ml O_2/g x h during (true) free flight, but only 21 ml/g x h during fixed flight, in the sphinx moth, *Manduca sexta* (see also review by Casey, 1989). The difference between the values for the two flight methods underlines the energy expense of generating lift which, according to the present data (Fig. 14), accounts for half of the energy required.

From a biochemical perspective, the qualitative changes seen during flight with and without lift generation are comparable. Although lift generating flight is the method of choice for approaching natural conditions, investigations during roundabout flight may also have merits, although lift and wing loading are substantially diminished and natural flight conditions are not reached. The advantage is that metabolic events are more protracted and can, therefore, be more easily investigated and interpreted. If, for example, a researcher wanted to analyse the fuels used for flight by a certain insect, roundabout flight would be the method of choice. If, however, the goal is to study maximal fuel utilisation in flight as it probably occurs during natural flight, then flight which includes lift production should be analysed in the laboratory and great care taken to distinguish between pre-flight warm-up, the first minute of flight and later phases.

It was assumed that during flight on the flight mill, when lift is apparently minimised, muscles involved in generating lift should show a slower depletion of substrates compared with lift generating flight. Therefore, metabolic changes were measured separately in three different muscles of the flight apparatus (see Fig. 2, section 2.2.1.). As expected, metabolic changes during lift generating flight in the lift-generating downstroke muscles (BM and DLM) and in the DVM, one of the upstroke muscles responsible for producing drag, are not different, because all these muscles are used. However, during roundabout flight, no large differences were found, either. Unexpectedly, the DVM was found to have used less proline and glycogen and generated less alanine than the two downstroke muscles. These results suggest a major participation of the downstroke muscles, BM and DLM, in generating both lift and drag. This pattern of substrate use, in addition to the similar resting values of metabolites in the various muscles, excludes a biochemical specialisation of certain muscles to one or the other fuel.

Using substrate utilisation rate and the equation for the partial oxidation of proline the contribution of the different fuels (carbohydrates and proline) as well as the contribution of the major compartments (flight muscles, haemolymph) to the overall energy consumption for a "model beetle" (as outlined in 3.1.8.) during 30 s of lift generating flight can be calculated. As shown in Table 23, proline and carbohydrates make similar contributions to the energy needed for flight (43.4 versus 56.6 %, respectively). A major part of flight substrates in *P. simuata* is supplied by the flight muscle tissue itself (54 %; Table 23). The haemolymph also supplies a large amount of energy (46 %), but this is relatively less than what was found in the blister beetle, *Decapotoma lunata* (75 %). The difference is mainly caused by the fact that *P. simuata* has a much lower relative haemolymph volume (163.7 µl/gfw) than *D. lunata* (347.2 µl/gfw). The sum of the two compartments amounts to a mass specific oxygen consumption of 95.2 ml/g x h in *P. simuata*. Because the metabolic changes in fat body were not measured during lift generating flight, it was assumed, from the data obtained for flight on the flight mill, that the rate of substrate depletion in fat body is similar to that of flight muscle tissue. When the rate of re-synthesis of proline is also taken into account (see Fig. 6A and 7A for rest after flight values), V_{O_2} is well above 100 ml/g x h, despite the fat body's low percentage of the body mass. This value is in accordance with results obtained from measuring V_{O_2} *in vivo* (about 105 ml O_2/g x h, Fig. 13) however, it is high compared with other flying scarab

Table 23. Oxygen consumption calculated from data on substrate utilisation during free flight of *P. sinuata* and *D. lunata* .-

		Consumption of substrate during flight*		molar ratio	O_2 consumption (µmol/gfw x min)	ml O_2/gfw x h	(ml O_2/g beetle x h)		
P. sinuata	Flight muscle		µmol/gfw/min						
		Proline	31.6	2.5	79	106.2	8.7	17%	
		Glycogen	64.3	6	385.8	518.5	42.5	83%	
							51.2		53.8%
	Haemolymph		µmol/ml/min						
		Proline	60.2	2.5	150.5	202.3	33.6	90.6%	
		Carbohydrates	7.77	6	46.6	62.66	10.4	23.6%	
							44.0		46.2%
							95.2		100%
D. lunata	Flight muscle		µmol/gfw/min						
		Proline	2.35	2.5	8.4	11.3	2.71	41.2%	
		Glycogen	2.0	6	12	16.1	3.86	58.8%	
							6.57		24.2%
	Haemolymph		µmol/ml/min						
		Proline	0.69	2.5	1.73	2.33	0.88	4.3%	
		Carbohydrates	6.44	6	38.6	51.9	19.65	65.7%	
							20.53		75.8%
							27.1		100%

*Metabolite changes during the first 30 s of flight of *P. sinuata* and the first 10 min of flight of *D. lunata* were taken.
The difference for *P. sinuata* was always taken from warm-up level, except for haemolymph carbohydrates (decrease of concentration between 1 and 5 min of flight taken)
Data taken from Fig. 6 and 7 (for *P. sinuata*) and 16 and 17 (for *D. lunata*) .

beetles such as the green fig beetle, *Cotinus texana* (45-90 ml O_2/g x h; Chappell, 1984) or the Japanese beetle, *Popillia japonica* (52 ml O_2/g x h; Örtli and Örtli, 1990).

4.1.5. Patterns of metabolic changes during starvation

During the life span of *P. sinuata* weather conditions can occur which make it impossible for the insect to fly and reach its food sources. In October, for example, when the beetles are already active, cold and rainy periods of up to 10 days, with temperatures as low as 15 °C, are still possible. Such temperatures are too low for *P. sinuata* to fly (Heinrich and McClain, 1986) and the beetles would starve for several days during such a period. Therefore, investigations were undertaken to observe the metabolic situation during starvation in the fruit beetle. In addition, such experiments can give information which can be compared with the metabolic changes during flight activity, because starvation, like flight, leads to a net loss of energy substrates such as glycogen.

For example in *L. migratoria*, metabolite changes caused by starvation and flight were found to be similar. Haemolymph carbohydrate concentration dropped by more than 50 % during 30 min of flight (Jutsum and Goldsworthy, 1976), while it fell by nearly 90 % during starvation of 96 h (Jutsum *et al.*, 1975). During 3 h of locust flight, the haemolymph lipid concentration increased roughly 2.5-fold (Jutsum and Goldsworthy, 1976), while during starvation of 96 h the lipid level is elevated two to five-fold (Jutsum *et al.*, 1975; Cheeseman and Goldsworthy, 1978). Similar changes were found during flight (Ziegler, 1986a,b) and starvation (Ziegler, 1991) of adult moths, *M. sexta*.

The metabolic pattern during starvation of *P. sinuata*, however, is different in this respect. While carbohydrates stores, similar to locusts, in haemolymph, flight muscles and fat body were drastically reduced, proline concentration decreased only slightly even after a very long starvation period of 30 days. Alanine disappeared almost completely during this time. After re-feeding of beetles, which had starved for 30 days, the trend was reversed and carbohydrate and alanine concentrations increased rapidly within the first 2 h. Proline concentration, however, remained at the pre-feeding level.

Weeda *et al.* (1979) measured metabolite changes in the Colorado potato beetle during a shorter starvation period of 5 days. Changes in the haemolymph, comparable with those of the early period of starvation in *P. sinuata* in the present study, were found with the carbohydrate concentration decreasing by roughly 50 % in both beetle species,

while no changes were recorded in lipid levels. A slight increase of proline concentration was apparent in the Colorado beetle as well as in the fruit beetle.

It is difficult to compare the metabolite changes in flight muscles and fat body of the two beetles, due to the fact that in the present study these changes were measured after a period of 15 days of starvation (Fig. 26) compared with the 5 days examined by Weeda *et al.* (1979). However, the glycogen content, which decreased to nearly 15 % during 15 days of starvation in *P. sinuata* flight muscles, showed no marked change in *L. decemlineata* flight muscles. In the flight muscles of both beetles the proline and alanine content remained virtually unchanged. In both, *P. sinuata* and *L. decemlineata*, an increase of proline and a slight decrease of alanine concentration seemed to have occurred during the early starvation period. As in *P. sinuata*, the stable proline concentration during starvation in *L. decemlineata* is in contrast to the rapid decrease during flight.

The question arises, why proline, which was shown to be an important energy source for flight (see sections 3.1.1., 3.5.), is kept at this high level, while the carbohydrates, the other major fuel in *P. sinuata*, are almost completely consumed?

The carbohydrate content of the haemolymph obviously depends very much on the amount of nutrients released from the gut into the haemolymph. This could also be shown for the blister beetle, in which the carbohydrate concentration in the haemolymph already decreased by 35 % overnight when not fed (see 2.2.2.).

Alanine is not only the end product of partial proline oxidation but also a substrate for gluconeogenesis. This was shown in fed and starved adults of *Tenebrio molitor*, where ^{14}C-alanine was rapidly incorporated into carbohydrates (Gourdoux *et al.*, 1984). In starved animals the incorporation rate was doubled compared with fed animals. This enables the insect to re-distribute carbon units from the amino acid metabolism into carbohydrate metabolism and may be important during starvation for keeping the glycogen and glucose content at a minimum level in vital organs such as the brain. The disappearance of alanine from *P. sinuata* haemolymph (Fig. 25A) may be attributed to such a re-distribution.

Proline, however, remained at high levels during starvation comparable to those in fed beetles. It was shown that proline served as the fuel to power pre-flight warm-up in *P. sinuata* (see 3.2.2.) and is as such the initial fuel.

Fruit beetles seemed to fly exclusively on proline after prolonged starvation. In all organs investigated, almost no carbohydrates were measured (see Fig. 26). In addition, injection of Mem-CC (Table 4) could not elevate the haemolymph carbohydrate level (Lopata and Gäde, 1994), indicating that all reserves had been depleted. Despite this lack of carbohydrates, beetles were still eager to fly and were able to generate lift for 3-6 min after 30 days of starvation. This is in contrast to starved locusts whose flight ability is poor during starvation (Goldsworthy and Coupland, 1974).

The above results led to the conclusion that carbohydrates, although a major flight substrate, were not essential for flight in *P. sinuata*. In contrast, proline seemed to be a pre-requisite for flight capability of this beetle. To retain flight capability when food cannot be reached, concentrations of proline are maintained at a high level. To reach food sources after starvation under natural conditions is possibly a question of survival. Therefore, the beetle must be able to warm-up if the ambient temperature is not sufficiently high. The availability of proline is essential for this warm-up (see 4.3.1.), as carbohydrates cannot perform this function. Carbohydrates can therefore be subject to consumption for resting metabolism or other activities not related to flight during starvation. The beetles avoid flying in cold weather, although they would theoretically be able to. This is probably due to the high energetic cost of warm-up.

This special function of proline and the capability of the beetle to preserve its high concentrations during food deprivation were probably helpful for the beetle to adapt to changeable conditions and therefore supported its distribution throughout South Africa (see Introduction).

4.1.6. Lipids - a fuel for *P. sinuata* flight?

During both methods of flight experiments with *P. sinuata* an increase of haemolymph lipid levels was observed (sections 3.1.1. and 3.1.2.). In addition, the activity of flight muscle HOAD, an enzyme representing the fatty acid break down, was relatively high (Table 5). These observations might suggest that lipids were used to power the flight of the beetle. However, in contrast to these were some other findings. Firstly, the lipid increases of 1.5 mg/ml after 11 min of lift generating flight and 9.1 mg/ml after two hours of roundabout flight were small compared with those occurring in *L. migratoria* during 30 min of tethered flight (about 15 mg/ml; Goldsworthy *et al.*, 1972). A small increase in haemolymph lipids similar to *P. sinuata* was observed in the

Colorado potato beetle, *Leptinotarsa decemlineata*, during tethered flight of 20 min (Mordue and de Kort, 1978) and 10 min (Weeda *et al.*, 1979).

Secondly, unlike in locusts, where the haemolymph lipid levels continued to increase during subsequent rest after flight (Jutsum and Goldsworthy, 1976), the concentration remained unchanged during rest following flight in the fruit beetle (sections 3.1.1. and 3.1.2.). Additionally, the haemolymph lipid concentration was unchanged by starvation in *P. sinuata* (Fig. 25), in contrast to the situation in *L. migratoria* where lipid levels rose (see previous section). Moreover, if lipids were used, one could expect that the endogenous AKH-peptide of an insect would initiate the release of lipids from the fat body as it is known from locusts (Goldsworthy, 1983; Gäde, 1996, 1997c). Such an effect of Mem-CC, the beetle's own AKH-peptide, could not be shown (Table 14; Lopata and Gäde, 1994). During the entire time course of flight the RQ was 0.9. However, when lipids were involved in energy supply a RQ closer to the theoretical value of 0.7 for lipid oxidation could be expected. Finally, the capability of *P. sinuata* flight muscle mitochondria to oxidise palmitoyl-carnitine was poor compared with that of locust mitochondria (Table 7). The sum of the above observations supports the conclusion that lipids were not oxidised by *P. sinuata* flight muscles during flight.

4.2. Pathway of proline metabolism

4.2.1. Proline oxidation during flight preparation and flight

During endothermic pre-flight warm-up, the proline concentrations in haemolymph and flight muscles declined steeply, while alanine started to accumulate (see 3.2.2.). This trend continued during the first phase of flight until the concentrations of both amino acids reached steady state in the second phase (discussed in 4.1.4.). From the results of pre-flight warm-up and the first flight period it can be seen that the changes of proline and alanine are roughly equimolar. The same can be stated for the blister beetle and the Protea beetle (see 3.1.4. and 3.1.5.). A similar feature of proline breakdown was reported for the tsetse fly (Bursell, 1963; Hargrove, 1976) and for the Colorado potato beetle (Weeda *et al.*, 1979).

The accumulation of alanine indicated the presence of a functioning transamination system. Indeed, it was shown that *P. sinuata* possessed the enzymatic pre-requisites for

the partial oxidation of proline, namely sufficiently high activities of AlaT and ME (Table 5).

The first step of proline breakdown is mediated by the proline dehydrogenase (ProDH) which catalyses the conversion of proline to pyrroline-5-carboxylate which then converts spontaneously to glutamate-5-semialdehyde. The latter intermediate is subject to oxidation by the glutamate semialdehyde dehydrogenase. The carbon moiety of the formed glutamate can theoretically be introduced into the Krebs cycle as α-ketoglutarate by the action of glutamate dehydrogenase (GluDH). This reaction, however, would lead to the release of ammonia which is thought to inhibit the GluDH in insect flight muscles (Mordue *et al.*, 1980). This was shown for the GluDH of tsetse fly flight muscles (Bursell, 1975). In addition, the carbon skeleton would be completely oxidised when this pathway was used. Although there were some indications that at least a portion of the proline was broken down via this pathway in the tsetse fly (Hargrove, 1976) and the Japanese beetle (Hansford and Johnson, 1975), the major route in these insects was shown to be the transamination catalysed by the action of alanine aminotranferase (AlaT) (Bursell *et al.*, 1974; Hansford and Johnson, 1975). Because there is no release of ammonia during the course of this pathway, it has a clear advantage over the GluDH pathway. No additional mechanisms are required to handle excess ammonia. A pre-requisite for the functioning of this pathway, high activity of AlaT in the flight muscles, was confirmed for *P. sinuata* (see section 3.4.1.). However, the activity of the GluDH is also high. In the blister beetle, both enzymes have similar levels of activity. However, the accumulation of alanine in the body during flight confirmed that the AlaT pathway was most likely the major route of proline catabolism. When the pathway via the AlaT is used, pyruvate is necessary as an acceptor for the ammonia of glutamate. A pre-requisite of that pathway is therefore an enzyme which removes a carbon moiety from the Krebs cycle and provides pyruvate for the transamination (Bursell, 1963). This function is fulfilled by the NAD-dependent malic enzyme (ME), found to have high activities in flight muscles of insects which use the partial oxidation of proline (see Bursell, 1981). The activity of the ME must be sufficiently high to ensure the removal of malate from the Krebs cycle despite the presence of a strong MDH which uses the same substrate as the ME (Bursell, 1981). Therefore, Bursell (1981) proposed the MDH:ME ratio to describe the importance of the partial proline oxidation in the flight muscles of an insect. According to this measure, the pathway is best developed in the tsetse fly, where a ratio

of 1 was found (Hoek *et al.*, 1976). Ratios of 13 in *P. sinuata* and 7 in *D. lunata* are similar to the ratio of 8 found in *L. decemlineata* (Weeda *et al.*, 1980a).

Competition of the two enzymes for malate *in vivo* seems unlikely though. During the exclusive use of proline in the tsetse fly, the action of MDH would create a deficit of acceptor pyruvate which in turn would cause accumulation of ammonia. Weeda (1981b) explained that the two enzymes were not active simultaneously *in vivo*, because at a neutral pH the activity of MDH was negligible in Colorado beetle flight muscles. However, this would also exclude any oxidation of carbohydrates, which are a major flight substrate in this beetle (Weeda *et al.*, 1979).

Besides the use of the pathway of partial oxidation, a portion of glutamate might be deaminated by GluDH and the resulting α-ketoglutarate completely oxidised. Indications that 20 % of the proline was completely oxidised at the onset of flight of the tsetse fly have been found (Hargrove, 1976). However, no direct indications for such a scenario were observed with the experimental set-up in the present study. A highly active GluDH is present, however, in the flight muscles of *P. sinuata* and after 30 s of lift generating flight and 15 min of roundabout flight a markedly lower sum of haemolymph proline and alanine was observed (see 3.1.1. and 3.1.2.). These may be indications that at the begin of flight the action of GluDH created a deficit of amino acid nitrogen.

However, during *in vitro* oxidation of proline by isolated flight muscle mitochondria the formation of alanine was equimolar (see 3.5.). From these results it can be calculated that 2.42 mol O_2 were consumed during the oxidation of 1 mol proline or 2.3 mol for the production of 1 mol alanine. This suggests that proline is catabolised in *P. sinuata* flight muscles using the pathway of partial oxidation. During the course of this route 14 mol ATP can be generated (Bursell *et al.*, 1974). The overall equation for proline breakdown should therefore be as follows:

$$\text{proline} + 2.5\ O_2 + 14\ \text{ADP} \rightarrow \text{alanine} + H_2O + 2\ CO_2 + 14\ \text{ATP}$$

Partial oxidation according to the above equation was first discovered in the tsetse fly (Bursell *et al.*, 1974) and later in the Colorado potato beetle (Weeda *et al.*, 1980a). These results of experiments *in vitro* still need to be confirmed for the situation *in vivo* in *P. sinuata* by means of radiolabelled precursors.

Although oxidation of proline requires only a part of the Krebs cycle (from α-ketoglutarate to malate), the remaining section seems to be equally developed as can be

expected from the utilisation of carbohydrates by *P. sinuata* flight muscles during flight activity. An indication of this are high activities of citrate synthase (Alp *et al.*, 1976; Zebe and Gäde, 1993) and malate dehydrogenase (present study). These data resemble those measured in the Colorado potato beetle (Khan and de Kort, 1978; Weeda *et al.*, 1980a). In tsetse flies, however, that section of the Krebs cycle which is not involved in partial proline oxidation is less developed. This can be concluded from the low activities of citrate synthase, malate dehydrogenase, aconitase and isocitrate dehydrogenase (Norden and Matanganyidse, 1979). The low enzyme activities of this section of the cycle are in agreement with the fact that tsetse flies contain only negligible amounts of carbohydrate reserves and do not use carbohydrates as fuel for flight (see Bursell, 1981).

4.2.2. The synthesis of proline

4.2.2.1. The fat body - the place of proline synthesis

As in the tsetse fly (McCabe and Bursell, 1975; Bursell, 1977) and the Colorado potato beetle (Weeda *et al.*, 1980b), the fat body of *P. sinuata* was found to be the site of proline synthesis as well (Table 16).

The enzymes thought to be necessary for proline synthesis were present in the fat body of *P. sinuata*. Activity of AlaT was high, while its activity was negligible in locust fat body (Table 6). Previously, high AlaT activities have been reported for fat body tissue of tsetse flies (Konji *et al.*, 1984) and the Colorado potato beetle (Khan and de Kort, 1978; Weeda *et al.*, 1980b). In those insects it is believed that AlaT is responsible for translocating the amino group from alanine to α-ketoglutarate to form glutamate for subsequent reduction to proline (see Fig. 1).

The activities of the NADP-dependent ME in the fat bodies of the latter two insects was also high, while the NAD-dependent ME had negligible activities. In the present study the same situation was found in *P. sinuata* fat body, while activities of NAD- as well as NADP-dependent ME of locust fat body were low (Table 6). The NADP-dependent ME of fat body tissue is thought to carboxylate the pyruvate resulting from transamination (see Fig. 1).

The fat body of *P. sinuata* also exhibited high HOAD activities. A functioning HOAD is a pre-requisite for the supply of carbon moieties from the β-oxidation of fatty

acids via acetyl-CoA (see Fig. 1). The above mentioned authors found this to be the same in the tsetse fly and the Colorado potato beetle.

4.2.2.2. Precursors for proline synthesis

Fat body tissue of *P. sinuata* was found to synthesise proline with alanine as the sole substrate in the incubation medium (Table 17, Fig. 44). In addition, the consumption of alanine and the production of proline was shown to be equimolar (Table 20). Because alanine can only provide three of the required five carbon units, the remaining two must be supplied from an endogenous source. Experimental results excluded the possibility that one new proline molecule is formed from two alanine molecules according to the equation:

$$2 \text{ alanine} + NAD^+ \rightarrow \text{proline} + NH_4^+ + CO_2 + NADH + H^+,$$

which was proposed as one of two possibilities for proline re- synthesis in the tsetse fly by Bursell (1977). Despite the fact that this pathway would run out of alanine relatively early during flight, it would also leave one molecule of ammonia left per each molecule of proline synthesised. Its elimination would require additional energy. The second possibility for proline re-synthesis was expressed by Bursell (1977) by the equation:

$$\text{alanine} + \text{acetyl - CoA} + NADH + H^+ \rightarrow \text{proline} + 2\ H_2O + NAD^+ + \text{CoA}.$$

The acetyl-CoA would deliver the two carbons necessary in addition of those from alanine, which also supplies the amino group via transamination. No excess release of ammonia would occur during proline synthesis following the latter pathway. This raises the question as to where acetyl-CoA is derived from. Many metabolic pathways can supply substrates for the Krebs cycle via acetyl-CoA. One of these pathways supplying metabolites in sufficient quantities is glycolysis. Therefore, glucose was tested as a substrate in an incubation experiment, however, no substantial proline production was observed (Table 17). In addition, from the results of the flight experiments during a starvation period it can be concluded, that proline re-synthesis is also possible when all carbohydrate reserves are depleted (Fig 27) and, thus, glycolysis is of minor importance in supplying acetyl-CoA. The high activities of two enzymes of carbohydrate breakdown, GAPDH and glycogen phosphorylase, therefore seem not to be involved in the proline re-synthesis.

Carbon from several amino acids can enter the Krebs cycle via acetyl-CoA, for example leucine and tyrosine. Both substrates were tested in different incubation

experiments and it was shown that neither of them can contribute to the synthesis of proline in the fat body of *P. sinuata* (Tables 17 and 18). While no proline synthesis took place when fat body was incubated in medium which contained leucine, tyrosine could be excluded as a carbon source, because no radiocarbon of labelled tyrosine could be included into proline even when alanine as a precursor was supplied. Radiocarbon of aspartate was strongly incorporated into proline in the presence of alanine, while only a little of the label appeared in proline when no alanine was provided. Although it is theoretically possible that aspartate can supply C_2 units, it is unlikely, because this amino acid is not present in *P. sinuata* in sufficient quantities to meet the demands during flight (section 3.1.2.).

In contrast to the tsetse fly and the Colorado potato beetle (Khan and de Kort, 1978; Konji *et al.*, 1984), GluDH had negligible activity in the fat body of *P. sinuata* and therefore cannot play a substantial role in the re-synthesis of proline.

Another major pathway which is connected with the Krebs cycle via acetyl-CoA is the β-oxidation of fatty acids. Universally labelled palmitate was therefore tested as a possible supplier of the two carbon units. During these experiments it was confirmed that fatty acids cannot feed the proline synthesis as a sole carbon source (Tables 18 and 22). If alanine is present in the medium, however, radiocarbon was rapidly incorporated into proline (Tables 18 and 22), confirming both the essential role of alanine as a precursor and the ability of the β-oxidation to contribute the two remaining carbon units.

During the course of the incubation experiments palmitate was taken up by the fat body cells and its radiocarbon was mainly incorporated into triglycerides (Fig. 45; section 3.7.4.). Radioactivity measured in the non-lipid phase and in some lipid fractions other than triglycerides such as free fatty acids (Fig. 45, section 3.7.4), is not sufficient to explain the amount of radiocarbon incorporated into proline (Table 22). It was therefore concluded that the majority of C_2 units for condensation with the carbon moiety of alanine derived from the triglycerides of *P. sinuata* fat body.

4.3. Regulation of flight metabolism in *P. sinuata*

The transition from rest to flight in an insect requires the establishment of a completely different level of metabolism with the metabolic rate increasing dramatically. In the case of *P. sinuata*, oxygen consumption was elevated from 1.3 to 104 ml/g x h during lift generating flight (see section 3.1.4.). During rest-flight transition and during

the early phase of flight, regulation of the metabolic flux is necessary at various levels to organise the oxidation of substrates as well as the supply of additional substrates from the fat body.

4.3.1. Regulation of flight metabolism at physiological level

4.3.1.1. Flight preparation (importance of thoracic temperature)

In many insects, flight is only possible when certain flight muscle temperatures are reached (see Heinrich, 1993). This was shown to be the case in *P. sinuata* (Heinrich and McClain, 1986; section 3.2.1.). *P. sinuata* was described as "lazy" in terms of its maintained low body temperature during non-flight activities (hypothermia; Heinrich and McClain, 1986). The present results are in agreement with this earlier work. The beetle needs a thoracic temperature of 28 °C (Fig. 20) to fly effectively. Before flight, *P. sinuata* warms up endothermically to about 33 °C, if passive elevation of T_{th} by basking in the sun is not possible. This is similar to the closely related Protea beetle, *Trichostetha fascicularis* (Nicolson and Louw, 1980) but different from certain dung beetles, which maintain a high body temperature during terrestrial activity (Bartholomew and Casey, 1977; Bartholomew and Heinrich, 1978; Heinrich and Bartholomew, 1979). The relatively narrow range of T_{th} at the start of flight in *P. sinuata* is in contrast to *M. melolontha*, which is able to take off at thoracic temperatures between 19-30 °C (Schneider, 1980). This might be an expression of adaptation to different climatic conditions under which the two beetles have to fly. While *P. sinuata* lives at high T_a and can achieve the required T_{th} easily by basking in the sun, the cockchafer lives in a colder climate and during its flight activity in the dusk, exposure to sun is impossible. Thus, while it is possible for *P. sinuata* to avoid the high energetic costs of warm-up (see below) by basking, the cockchafer has to elevate its T_{th} prior to flight. The wide range of T_{th} for flight, which is probably based on a wider range of temperature optima of involved enzymes (see below), ensures therefore that the warm-up can be kept as short as possible in order to save energy. In addition, the life span of *M. melolontha* of only a few weeks is much shorter than that of *P. sinuata*, for which more than 200 days were reported (Lopata, 1991). Reaching flight ability with minimum energetic cost at a wide range of ambient temperatures is therefore essential to ensure reproductive success of the

cockchafer during this short period. In contrast, the fruit beetle with its longer life span is able to avoid such sub-optimal flight conditions.

Like *T. fascicularis* (Nicolson and Louw, 1980), *P. sinuata* is unable to preserve the generated heat (Heinrich and McClain, 1986). T_{th} stays constant when heat production and heat loss are in an equilibrium, which is at a T_a of around 30 °C. At lower values of T_a, T_{th} declines. Similar observations have been made in *M. melolontha* (Schneider, 1980). In the cockchafer, as in *P. sinuata*, wing beat frequency, and thus flight performance, is closely related to T_{th}. This influence of T_{th} on flight performance is probably caused by the properties of the enzymes involved in muscle contraction. Barnett *et al.* (1975) showed that flight muscle phosphorylase and actomyosin ATPase of *P. sinuata* have temperature optima at about 40°C, below which activity declines steeply.

Endothermic pre-flight warm-up requires large amounts of energy, as can be seen from the oxygen consumption rate during this period. The value measured (46.8 ml O_2/g x h) is in the same range as that for flight on a flight mill (43.8 ml O_2/g x h) and almost half of the amount which is consumed during lift producing flight (Fig. 14).

Unequivocal evidence was provided that proline supplies most of the energy required when elevation of T_{th} has to be achieved endothermically: while no changes occurred in haemolymph lipids and carbohydrates, proline concentration decreased steeply and alanine concentration rose concomitantly (Fig. 24). In addition, glycogen stores in the flight muscles seem unaffected by warm-up, while a dramatic drop of proline concentration occurred, accompanied by the concomitant elevation of alanine levels (Fig. 23). These data are supported by measurement of RQ. During flight, as well as at rest, RQ was around 0.9. During warm-up, however, the RQ was significantly lower at 0.82 (Fig. 15), which is close to the theoretical value of 0.8 calculated for the partial oxidation of proline (see review by Bursell, 1981).

Very few data are available on the biochemical changes occurring during endothermic warm-up in other insects. Joos (1987), who investigated the endothermic sphinx moth, *Manduca sexta*, found that carbohydrates are the energy source in this insect, which otherwise flies predominantly on lipids (Ziegler and Schulz, 1986b). Newsholme *et al.* (1972) proposed heat production by non-shivering thermogenesis by a substrate cycle of fructose-6-phosphate and fructose-1,6-diphosphate catalysed by the enzymes phosphofructokinase and fructose diphosphatase. In these experiments, however, it was not proven if the muscles were really at rest. Instead, action potentials,

and thus muscle activity, were measured during the warm-up period in different Hymenoptera such as *Apis, Xylocopa* and *Bombus* (Esch *et al.*, 1991). In the latter insect, the substrate cycle was found and its function for warm-up proposed (Newsholme *et al.*, 1972). In *P. sinuata* the involvement of such a cycle in endothermic warm-up seems unlikely. The RQ should be closer to unity during this period, when the cycle was present.

Because proline is a major fuel in the fruit beetle, these findings, that proline powers pre-flight warm-up in *P. sinuata*, support suggestions by Heinrich and McClain (1986) that heat production is achieved by muscle activity (shivering). Shivering of the flight muscles has already been observed by Krogh and Zeuthen (1941) in *Geotrupes stercorarius* and in *Acilius* (Leston *et al.*, 1965). Bartholomew and Casey (1977) found that a thermocouple vibrated when it was inserted into the thorax of two dung beetle species. Because no movement of wings and elytra are visible in *P. sinuata*, it is most likely that the flight muscles are activated synchronously (unlike during flight) to contract isometrically in order to achieve heat production but no power output. Kammer (1968) concluded that the synchronous activation of flight muscles during shivering derived from the asynchronous activation during flight and is therefore a modification of flight. The present results concerning the use of proline during warm-up support this theory.

Some insects, such as locusts, use only small amounts of proline shortly after the commencement of flight (Worm and Beenakkers, 1980) which probably serves a "sparker" function in order to provide the Krebs cycle with intermediates as proposed for the blowfly (Sacktor and Wormser-Shavit, 1966; Sacktor and Childress, 1967). The use of proline as a major or even the sole flight substrate in certain scarab beetles such as *P. sinuata*, *Onitis* and *Scarabaeus* (Gäde, 1997a,b) might have derived from its original function as a sparker. Its high resting concentration in flight muscles and haemolymph of the above beetles and the immediate availability of its carbon skeleton for the Krebs cycle also makes it an ideal fuel for warm-up. It would be interesting, to examine the function of proline in *Onitis* and *Scarabaeus*. In conclusion, it was shown that *P. sinuata* needs a T_{th} of at least 33°C to start flying voluntarily and that if this T_{th} is achieved actively by endothermic pre-flight warm-up, proline is the sole energy substrate.

4.3.1.2. Influence of metabolic neuropeptides

Insect flight is very energy demanding and the reserves immediately available from stores in the haemolymph and flight muscles are rapidly depleted. As can be seen from section 3.1.1., haemolymph proline and flight muscle proline and glycogen are diminished to about 50 % of the resting levels within the first 30 s of flight. Fuels from other sources, namely the fat body, have to be mobilised to meet the demand during long-term flight. In locusts lipid stores of the fat body are made available (see Goldsworthy, 1983) while in cockroaches the glycogen of the fat body is broken down and the resulting trehalose released into the haemolymph (see Beenakkers *et al.*, 1984). From locusts it is well known that this process is under the control of neuropeptides of the AKH/RPCH family, which are synthesised and stored in the intrinsic cells of the corpora cardiaca and released from there into the haemolymph during flight (see Gäde, 1996; 1997c).

As discussed earlier, proline is an important flight substrate in some insects and there are some indications that AKH peptides are involved in the regulation of its metabolism during flight. An investigation *in vitro* with fat body preparations of *L. decemlineata* demonstrated that crude extract of corpora cardiaca is capable of increasing the release of proline from the fat body, while it also leads to a quicker disappearance of alanine from the medium (Weeda, 1981a). In the same study it was shown that synthetic Lom-AKH-I had the same effect when added to the incubation. However, because the beetle's own AKH peptides, Pea-CAH-I and II, were only discovered and fully characterised later (Gäde and Kellner, 1989), the stimulation of proline synthesis could not be linked conclusively to the endogenous AKH peptides of *L. decemlineata*. Although alanine concentration in the haemolymph of the Colorado potato beetles was shown to decrease upon injection of corpora cardiaca extract of the beetles, locusts and cockroaches as well as Lom-AKH-I, no hyperprolinaemic activity *in vivo* with either substance was demonstrated (Weeda, 1981a). An increased proline release by isolated fat body cells, incubated with conspecific corpora cardiaca material, was also observed in the tsetse fly, *G. morsitans* (Pimley and Langley, 1982; Pimley, 1984). Only recently was it shown by Gäde (1997a,b) that conspecific injection of corpora cardiaca extract into onitine and scarabaeine dung beetles caused hyperprolinaemia *in vivo*. In contrast to findings in the tsetse fly, the latter author was able to demonstrate that this effect is caused by AKH/RPCH peptides separated by HPLC from the beetles' corpora cardiaca as well as by synthetic peptides.

The present study demonstrates that the corpora cardiaca of the African fruit beetle, *Pachnoda sinuata,* contain factors which cause an increase in the proline concentration in the haemolymph and a concomitant decrease in the concentration of alanine (Table 9). This confirms preliminary results mentioned by Lopata and Gäde (1994). Two of these factors were purified and subsequently one of these was shown to be Mem-CC. This peptide was first found and fully characterised in the cockchafer, *M. melolontha* (Gäde, 1991) and was later shown to be present in *P. sinuata* (Gäde *et al.*, 1992a). Conspecific injections of native peak material as well as synthetic Mem-CC confirmed the findings (Tables 9 and 10). The hypertrehalosaemic as well as hyperprolinaemic effect of Mem-CC was also shown when the endogenous source of the peptide was excluded by decapitation (Table 11).

The response of proline and alanine levels in the haemolymph of *P. sinuata* to injection of active material is time-dependent (Fig 35). The changes of proline and alanine concentrations were much quicker compared with those of haemolymph carbohydrates (Lopata and Gäde, 1994), but they were similar to the pattern of mobilisation of lipids and carbohydrates by the fat body of locusts and cockroaches by their own AKH peptides, respectively (Gäde, 1990). The maximum response of proline release by *L. decemlineata* fat body *in vitro*, however, was reached after 15 min of incubation (Weeda, 1981a).

The action of Mem-CC on the concentrations of the two amino acids is also dose-dependent. The maximum response of proline was achieved with the injection of approximately 25 pmol, which caused an increase of about 23 µmol/ml haemolymph. For the maximum decrease of alanine, about 7 µmol/ml haemolymph, only 15 pmol of the peptide was necessary. Taking the beetles' haemolymph volume of 166 µl (see 3.1.8.) plus the volume injected (10 µl) into account, 0.14 µM Mem-CC caused maximal hyperprolinaemia, while 0.09 µM Mem-CC was necessary for the maximal decrease in alanine concentration. These values are very similar to those for carbohydrate mobilisation in *P. sinuata* (Lopata and Gäde, 1994). The difference between the response of proline and alanine is probably caused by the low resting levels of alanine and, additionally, by the high resting levels of proline, and thus feedback inhibition (discussed in 4.3.2.).

The present study tried to answer the question, whether AKH-peptides with similar structures and from closely related insects (other beetles) have a comparable influence to

Mem-CC on the haemolymph concentrations of proline and alanine. Results obtained from injection experiments with different AKH peptides related to Mem-CC, and which have been found in other beetles, can give some information about the structural requirements of the receptor for Mem-CC in *P. sinuata*. In contrast to Gäde *et al.* (1992a) who found that position 4 (Tyr in this case) is most important for the ability to elevate haemolymph carbohydrate concentration of *P. sinuata*, the present findings clearly show that Tyr^4 is not essential for the effect on proline and alanine levels. Many peptides without Tyr^4 cause an effect similar to that achieved with Mem-CC. Important for receptor recognition is the amino acid in position 7. Of the 4 peptides with an amino acid in position 7 other than Asp or Asn, only Ona-CC (Gly^7) shows significant activity. Lom-AKH-III (Trp^7), Scd-CC-II (Val^7) and Ani-AKH (Ser^7) are not significantly active.

As already observed by Gäde *et al.* (1992a) for carbohydrates, no difference in activity on proline/alanine between peptides with Asp^7 (charged) or Asn^7 (uncharged) were found. The two additional amino acids of the decapeptide of the blister beetle, Del-CC, did not lower the response of concentrations of proline or alanine. However, it was found that the effect of Tem-HrTH and Pht-HrTH on proline and alanine is similar to that of Mem-CC, while these two peptides had a significantly lower activity than Mem-CC on carbohydrates in the beetle (Gäde *et al.*, 1992a). It is therefore very likely that the action of the peptides on proline and carbohydrate metabolism is mediated by different receptors.

4.3.1.3. Novel neuropeptides of cetoniine scarabs

Although their structure could not been fully elucidated, two novel AKH peptides of *P. sinuata* and *T. fascicularis* display some unique new features. First, they have some of the common characteristics of the AKH/RPCH family in that they are both octapeptides and their N-termini are blocked by a pyroglutamate residue. In addition, they both have a Trp^8. However, they also possess some properties which have not been found in other members of the family so far. Position 6 causes a gap during Edman sequencing and is most likely an amino acid with a post-translational modification. Such modifications were found in Cam-HrTH-I of the stick insect, *Carausius morosus*, where Trp^8 is glycosylated with a hexose moiety (Gäde *et al.*, 1992b; Gäde, 1997d) and in the cicada, *Platypleura capensis*, where the kind of modification is still unknown (Gäde and Janssens, 1994; Gäde, 1997d). In addition , there was no aromatic amino acid found in

position 4 either. The two novel peptides contain Leu^4 and Met^4, respectively. Although the two peptides differ from the predominant AKH peptide in *P. sinuata* and *T. fascicularis*, Mem-CC, in 5 positions, they show similar bioactivity in conspecific bioassays - hyperglycaemia and hyperprolinaemia (Table 14). They also seem to share the Mem-CC's weak potential to elevate haemolymph lipids in locusts and haemolymph carbohydrates in cockroaches (Table 14).

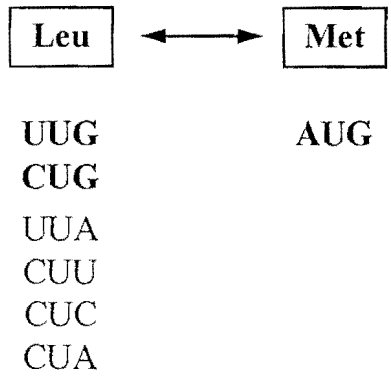

Figure 46. Possible mutational codon changes which have led to the amino acid exchange observed in position 4 of the novel peptides from *P. sinuata* and *T. fascicularis*.

It is noteworthy that the novel peptides differ very likely from each other only in position 4. Although the primary structure is not fully known yet, it can be assumed from the close phylogenetic relationship of the two beetle species and the unique situation that position 6 causes a gap during Edman sequencing, that position 4 is the only difference. That difference (Leu/Met) can be explained by a single base change in the genetic code (Fig. 46).

Although the novel peptide of *P. sinuata*, which was code-named Pas-CC-II according to the nomenclature proposed by Raina and Gäde (1988), was sequenced only from *P sinuata*, it can be assumed by comparison of HPLC retention times that *D. cincta*, *D. rufa* and *L. amethystina* contain the same peptide. The peptide sequenced from *T. fascicularis*, code named Trf-CC-II, also likely occurs in *T. albopicta*. These assumptions, of course, have to be proven by sequencing and fully characterising the structure of the peptides of the other species. It would appear that Pas-CC-II is a characteristic feature of the subtribe Cetoniina of the Cetoniini, to which the genera *Pachnoda, Dischista* and *Leucocelis* belong (Holm and Marais, 1992). Trf-CC-II, on the other hand, is present in two species of the genus *Trichostetha*, which is part of the

subtribe Diplognathina of the Cetoniini. More data need to be collected to prove whether these two peptides are characteristic of their respective subtribes.

While the two peptides are probably restricted to their respective subtribes, this is certainly not the case for Mem-CC. It has already been found in several members of the families Geotrupidae and Scarabaeidae of the super-family Scarabaeoidea. In the Scarabaeidae it was found in two subfamilies (Melolonthinae and Cetoniinae; Gäde, 1991; Gäde *et al.*, 1992a). This study adds to this list the presence in *C. innocua* (Melolonthinae) and the putative presence (assumed by comparison of HPLC retention times) in *Lepithrix* sp. (Melolonthinae) and *D. cincta, D. rufa* and *L. amethystina* (Cetoniinae).

4.3.1.4. Circadian rhythm of proline concentration

The proline concentration in the haemolymph in *P. sinuata* follows a clear circadian pattern (3.6.1., Table 37). Because the highest proline concentration can be measured in *P. sinuata* in the morning hours, when T_a is still low but when also the highest flight activity occurs, the reason might be an elevated proline synthesis caused by an endogenous release of Mem-CC to make sufficient substrate for flight available. The additional proline might also be necessary to ensure the ability of the animals to endothermically warm up, if ambient temperatures are not sufficiently high (discussed in 4.3.1.).

4.3.2. Regulation of flight metabolism at the cellular- and sub-cellular level

Regulatory factors on the physiological level such as thoracic temperature and possible hormones were discussed above. These phenomena have their basis in metabolic control mechanisms at the cellular level or below. Some of them are discussed further in a separate chapter.

4.3.2.1. Co-oxidation of proline and pyruvate in mitochondria

Respiration rates of isolated flight muscle mitochondria with proline and pyruvate as substrates are additive only when proline is present in low concentration. At high concentrations of proline the oxidation of pyruvate seems to become negligible, even when the maximum respiration, which was shown with proline, is not reached. That

would be the case *in vivo* when the animal is at rest. As soon as the pre-flight warm-up or flight starts, proline concentration declines dramatically. Because the proline has to pass at least the cell membrane and the inner mitochondrial membrane (the outer membrane is permeable), it is most likely that a steep gradient is created immediately after any flight muscle activity starts. The proline concentration should therefore drop rapidly to a level which enables pyruvate to participate in the energy supply for flight. Such a situation should be reached within seconds of flight with lift or, if the beetle warmed up endothermically, at the end of the flight preparation period. The increase of haemolymph carbohydrate concentration shortly after the onset of lift generating flight (see 3.1.1.) might be a result not only of the release of substrates into the haemolymph by the fat body, but also of the limited ability of the flight muscle mitochondria to oxidise pyruvate at this time of flight. This suppression of pyruvate oxidation and, e.g. glycolysis, by high proline concentrations might explain the RQ of 0.82 which was measured during the endothermic pre-flight warm-up and indicates the exclusive oxidation of proline. A decrease of the RQ because of the participation of fatty acid oxidation is unlikely in light of the poor capability of the flight muscle mitochondria to oxidise palmitoyl-carnitine (Table 7). During flight the RQ was 0.9 and indicates co-oxidation of proline and carbohydrates. However, the phenomenon does not explain the resting RQ, which is also 0.9. During rest proline levels in all organs measured were at their maximum. One could expect then a complete suppression of pyruvate oxidation and thus an RQ of close to 0.8 (see 4.2.1.). At rest the metabolic flux is low and may therefore allow a co-oxidation of proline and carbohydrates.

Similar to the above observations in *P. sinuata* flight muscle mitochondria, Weeda *et al.* (1980a) demonstrated an almost complete suppression of the pyruvate respiration by proline in mitochondria from flight muscles of *L. decemlineata*. The authors assumed that co-oxidation does not occur *in vivo*. They proposed that the pyruvate deriving from haemolymph carbohydrates and flight muscle glycogen was used as amino acceptor *in vivo*. According to this theory, the pyruvate would never be oxidised and would therefore accumulate. This seems unlikely.

In contrast to the inhibitory role of proline on pyruvate respiration in the beetles are the results of Sacktor and Childress (1967) using the blowfly, *P. regina*. They observed that proline stimulates pyruvate oxidation. However, in these insects proline is not a major flight substrate.

During flight of the locust a competitive inhibition of trehalose by diglycerides occurred in such a way that trehalose utilisation is inhibited as soon as the level of diglycerides in the haemolymph increased (Robinson and Goldsworthy, 1976). This inhibition might also take place at the mitochondrial level because it was not found when 2-bromostearic acid was administered (Jutsum and Goldsworthy, 1977), a specific inhibitor of the mitochondrial carnitine acyl transferase system of mammals (Chase and Tubbs, 1976). The competitive inhibition of trehalose breakdown by diglycerides was changed to a non-competitive inhibition through the action of AKH peptides (Robinson and Goldsworthy, 1976; 1977). Such a regulatory action on substrate preference by AKH peptides can not be excluded for the co-oxidation of proline and pyruvate in *P. sinuata* and *L. decemlineata*. This provides an interesting challenge for future research.

4.3.2.2. Feedback inhibition of proline synthesis

Results of bioassays with injection of AKH peptides and alanine as well as the observations concerning the circadian pattern of the proline concentration in the haemolymph demonstrated that the maximum level of proline is at around 120 µmol/ml (see sections 3.6.1. and 3.6.2.). As can be seen from Fig. 36, the pre-injection level of alanine seemed to be less important than that of proline for the maximum response of proline concentration upon injection of active material. Such results can be best interpreted by a feedback inhibition of proline synthesis. Even when alanine is injected into the beetles in addition to bioactive material, the maximum proline concentration cannot be elevated. The presence of a feedback inhibition seems also likely when one compares the maximum increase in proline levels by about 20 % of the pre - injection level with the increase of haemolymph lipids in locusts by more than 300 % (Gäde, 1990a) and haemolymph carbohydrates in cockroaches by almost 200 %. Even carbohydrate mobilisation by Mem-CC in *P. sinuata* amounted to an increase of about 100 % (Lopata and Gäde, 1994). When an incubation experiment was conducted in order to investigate the influence of proline on its own synthesis, such a feedback inhibition could be confirmed *in vitro* (Table 17).

A similar feedback inhibition was found during experiments *in vitro* by Bursell (1977) in the fat body of tsetse flies and by Weeda (1981a) with fat body of the Colorado potato beetle. The site of inhibition of proline synthesis is probably the transamination system: Bursell (1981) showed that the transamination of alanine by AlaT of tsetse fly fat

body was inhibited by proline. Feedback control of proline synthesis was also found in bacteria (Adams, 1970). The site of inhibition is probably the glutamyl kinase, which catalyses the reaction from glutamate to γ-glutamyl phosphate, a step during proline synthesis. This enzyme is known to be sensitive to low concentrations of proline in *Escherichia coli* (Baich, 1969) and in *Pseudomonas aeruginosa* (Krishna and Leisinger, 1979).

4.3.2.3. Site of action and mediation of signal of Mem-CC

Assuming a hormonal role of Mem-CC, questions arise as to what metabolic reactions are influenced and how the signal of Mem-CC is mediated from the receptor to the site of action. It was shown in sections 3.7.2. and 3.7.4. and discussed in section 4.2.2., that alanine and acetyl-CoA (deriving from lipid oxidation) are precursors for the synthesis of proline in the fat body. Consequently, the two possible sites of action are the pathway of alanine transamination and the lipid breakdown. Because injection of various concentrations of alanine as well as incubations of fat body tissue *in vitro* with alanine added to the medium have shown that the rate of proline synthesis increases as soon as there is alanine available, it seems likely that alanine supply is only affected by flight activity or warm-up.

The lipid breakdown might therefore be a target of the action of Mem-CC to make acetyl-CoA available for condensation with the carbon moiety of the accumulating alanine during and after flight. It is also noteworthy in this context that there is a difference in the sum of alanine and proline before and after injection of bioactive material which is often higher than 10 µmol/ml (see 3.6.1.). It might therefore be possible that other sources besides alanine are mobilised at the early stages of flight to supply the necessary carbon moieties as well as the ammonia. Other amino acids would be suitable to fill this gap. Although present only in low concentrations, aspartate and glutamate might contribute (see Tables 17 and 18), and the low activities of GluDH and AspT found in fat body tissue (Table 6) are probably sufficiently high to catalyse the necessary reactions.

Various attempts have been made to discover the way of mediation of the hormonal signal of AKH peptides from their receptor to the site of action. Corpora cardiaca extract or purified fractions thereof are capable of elevating the cAMP level of locust fat body *in vitro* (Spencer and Candy, 1976) and *in vivo* (Gäde and Holwerda,

1976; Gäde and Beenakkers, 1977). Subsequently it has been observed that low concentrations of Lom-AKH-I can increase the cAMP level in the fat body of locusts *in vivo* (Gäde, 1979). As reviewed by Goldsworthy (1983), the cAMP probably stimulates a protein kinase as the starting point of an enzymatic cascade, which eventually activates a triacylglycerol lipase. Ca^{2+} is most likely involved in this process as the lipid mobilisation *in vitro* is Ca^{2+} dependent (Spencer and Candy, 1976).

Mobilisation of the glycogen reserves of cockroach fat body depends on Ca^{2+} and cAMP (McClure and Steele, 1981). A model of the mode of action was developed by Steele (1982) according to which the AKH peptide causes an elevation of cAMP levels in the fat body cells, which increases the permeability of the cell membrane for Ca^{2+}. The elevated Ca^{2+} level activates an enzyme cascade and leads finally to the activation of the glycogen phosphorylase.

Corpora cardiaca extracts from tsetse flies stimulate the release of fatty acids and proline from fat body cells of tsetse flies *in vitro* (Pimley and Langley, 1982; Pimley, 1984). The release is Ca^{2+} dependent and might be mediated by cAMP (Pimley, 1985). Therefore, the site of action of AKH peptides on proline synthesis might be the activation of lipolysis via Ca^{2+}. This would be similar to the mobilisation of fat body lipids in locusts. However, all these speculations still need to be experimentally investigated and the mode of transmission of the signal from the receptor of Mem-CC to the activation site presents an interesting challenge for future work especially in the light of the fact that the peptide activates carbohydrate- as well as proline metabolism probably via different receptors (see 4.3.1.).

4.4. The use of proline in insects

The oxidation of proline as a major flight substrate has been studied in more detail only in *G. morsitans, L. decemlineata* and *P. sinuata*. Results of various authors, investigating different metabolic parameters of insects, provide evidence that the use of proline as fuel is more widespread and not limited to the above insects. Most of these observations were reported from members of the Coleoptera (see for example Crabtree and Newsholme, 1970; Pearson *et al.*, 1979; Gäde, 1997a,b). With the present study details relating to a number of different species have been added to the knowledge in this field. It has been shown that the blister beetle, *D. lunata*, has the enzymatic design within the flight muscle mitochondria to partially oxidise proline. This was confirmed *in vivo* by

conducting flight experiments in which it was found that proline contributed to about 15 % to the overall energy expenditure (Table 23). Experiments with flight muscle mitochondria of the Protea beetle, *T. fascicularis*, provided similar results to those obtained from the closely related fruit beetle, *P. sinuata* (Table 7). The flight experiments also confirmed the major role of proline during flight in this beetle (Fig. 18) and also revealed similarities with the metabolic changes found in *P. sinuata*. Some other (melolonthine) scarabs, such as the Christmas beetle, *C. innocua*, and the monkey beetle, *Lepithrix* sp., also have the ability to oxidise proline at substantial rates (Table 7). Their haemolymph proline concentrations are comparable with those found in *L. decemlineata* and *D. lunata* (Table 24). Furthermore, other beetle groups also seem to use proline to power their flight. For example, the tiger beetle *Cicindela* sp. also contains relatively high proline concentrations in its haemolymph (Table 24) and preliminary results from flight experiments with the cerambycid beetle, *Ceroplesis aethiops*, shows that haemolymph proline concentration drops during flight, while alanine increases (unpublished, data not shown). All these results suggest that the oxidation of proline as an energy substrate during flight is quite common in various members of the Coleoptera and that the biochemical pathway of its degradation is partial oxidation.

However, in species such as the tenebrionid *T. molitor* and the buprestid *J. cirrosa*, proline occurs in similarly low concentrations in the haemolymph as previously found in the locust (Table 24). A major contribution of proline to the energy supply during flight activity in these beetles seems unlikely and it probably only sparks the Krebs cycle by supplying intermediates during the transition from rest to flight as proposed for the blowfly (Sacktor and Wormser-Shavit, 1967).

These findings also demonstrate that in the Coleoptera the importance of proline for flight differs widely. *T. molitor* and *J. cirrosa* mark the lower end of a scale in which the top end is occupied by some onitiine and scarabaeine dung beetles (Gäde, 1997a,b). Carbohydrates are stored in negligible quantities in flight muscles and haemolymph of the latter species.

A combination of proline and carbohydrates use has been found in many beetles investigated thus far (Weeda *et al.*, 1979; present study). One beetle species, however, seems to oxidise lipids directly during flight (*Dendroctonus pseudotsugae*; Thompson and Bennett, 1971), although the authors have not investigated the participation of proline.

Table 24. Haemolymph concentrations of proline, carbohydrates and lipids in several insects.

species	concentration of proline (µmol/ml)	carbohydrates (mg/ml)	lipids (mg/ml)
Coleoptera			
Pachnoda sinuata	96.0	9.7	8.7
Dischista cincta	106.3	6.3	6.7
Trichostetha fascicularis	99.5	12.5	6.1
Melolontha melolontha	75.2	8.2	6.2
Camenta innocua	34.9	13.1	7.1
Lepithrix sp.	37.1	9.2	4.8
Onitis aygulus	48.4 [1]	2.1 [1]	8.5 [1]
Scarabaeus deludens	76.9 [2]	0.3 [2]	1.4 [2]
Decapotoma lunata	34.8	20.1	10.5
Leptinotarsa decemlineata	66.0 [3]	2.7 [3]	7.5 [3]
Cicindela sp.	25.7	7.1	5.4
Julodes cirrosa	13.0		
Tenebrio molitor	10.6	9.0 [4]	13 [4]
Orthoptera			
Locusta migratoria	9.2	26.8	9.9
Dictyoptera			
Periplaneta americana	13.6	14.9	15.7
Homoptera			
Platypleura capensis	0	59.7 [5]	13.9 [5]
Odonata			
Orthetrum julia falsum	12.4 [6]	10.0 [6]	20.0 [6]
Hymenoptera			
Xylocopa capitata	7.3	36.7	0.7
Lepidoptera			
Hippotion eson	3.4	75.7 [7]	8.1 [7]

Values taken from [1]Gäde (1997a), [2]Gäde (1997b), [3]Weeda *et al.* (1979), [4]Gäde (1988), [5]Gäde and Janssens (1994), [6]Janssens (1995), [7]Liebrich and Gäde (1995).

Bursell (1981) proposed a number of key aspects of proline metabolism in order to compare different insects with respect to the extent of proline use. These aspects include the capability of isolated flight muscle mitochondria to oxidise proline and pyruvate, the activities of proline dehydrogenase and alanine aminotransferase as well as the relative activity of malate dehydrogenase and malic enzyme (MDH:ME ratio). From this and from the results presented here, it can be concluded, that for a first assessment about the

quantitative involvement of partial oxidation of proline, only the determination of proline concentration in the haemolymph and AlaT activity is required. The advantage of this is that haemolymph samples can easily be taken in the field and kept frozen for a long time. AlaT activity can also be determined from frozen tissue in a convenient optical test. Other parameters suggested by Bursell, such as the relative capacities of the mitochondria for oxidation of proline and pyruvate, require fresh tissue or are more difficult to determine. The latter is true for the ProDH for which no optical test is available.

High activities of the AlaT were observed in *G. morsitans* and *M. melolontha* (Crabtree and Newsholme, 1970), *P. japonica* (Hansford and Johnson, 1975), *L. decemlineata* (Khan and de Kort, 1978) and the dung beetles *Catharsius* sp., *Heliocopris dilloni* and *Scarabaeus* sp. (Pearson *et al.*, 1979). The present study suggests that high activities of AlaT in flight muscles of *P. sinuata* and *D. lunata* are in relation to the catabolism of proline during flight in those beetles. For *G. morsitans* and *P. japonica* the use of proline was confirmed by other methods (Hansford and Johnson, 1975; Bursell, 1981), and dung beetles (including some *Scarabaeus* species) were found to oxidise proline during flight experiments (Gäde, 1997a,b). The use of proline during flight in *L. decemlineata* was also demonstrated *in vivo*. (Weeda *et al.*, 1979). In the present study a high concentration of proline was found in the haemolymph of *M. melolontha* (Table 24) and all these data demonstrate that a high activity of AlaT is linked to the partial oxidation of proline during flight. In addition, these data reflect the common use of proline in insects and that its use can also differ widely. However, many insects probably have not the ability to oxidise proline during flight at all. An example of this may be the cicada, *Platypleura capensis*, which does not have any proline (or alanine) in its haemolymph and which has been shown to use carbohydrates during (true) free flight (Gäde and Janssens, 1994).

4.5. Advantages of the use of proline

The question of why proline is used as a fuel for flight was raised early on (Kammer and Heinrich 1978; Bursell, 1981). In the light of the apparent widespread use of proline, at least within Coleoptera, it seems even more important to find a satisfying answer.

Initial attempts were aimed at explaining the phenomenon as an adaptation to the bloodsucking behaviour of the tsetse fly (Bursell, 1963; Bursell *et al.*, 1974), but this hypothesis became obsolete when it was found that the phytophageous Colorado potato beetle also oxidises proline (Weeda *et al.*, 1979).

Because feeding behaviour is obviously not helpful in explaining the advantages of proline as a fuel for flight, Bursell (1981) compared the use of proline with those of other substrates. The high solubility of proline, which enables the accumulation of high concentrations in the flight muscles and the haemolymph, was proposed as a possible explanation. This solubility makes proline readily available at the onset of flight in similar fashion to carbohydrates. Leucine and isoleucine, amino acids which could provide an energy yield similar to proline are less soluble (see Bursell, 1981). The energy yield from proline (0.52 mol ATP/g) is only slightly lower in comparison to lipids (0.65 mol ATP/g) and even higher when compared with glucose (0.18 mol ATP/g; Bursell *et al.*, 1974).

According to the equations of proline oxidation and re-synthesis, the molecular sum of proline and alanine is constant. The present results of flight experiments show that this is indeed true (see 3.1.1. and 3.1.2.). This means that no osmotic changes occur that need to be compensated at additional energy cost. In addition, the equimolarity of proline and alanine allows the possibility to keep the sum of nitrogen constant and to avoid the release of ammonia.

Re-synthesis of proline from alanine and acetyl-CoA allows to benefit from the efficient storage of energy in form of lipids (see Bursell, 1981) without the presence of a lipoprotein carrier for diacylglycerides, such as is found in locusts (see Wheeler, 1989). Mobilisation of lipids from the fat body is relatively slow, and the energy demand between the beginning of flight and the arrival of sufficient lipids in the flight muscles of the locust is met by carbohydrates in the flight muscles and haemolymph. In the insects that use proline the period between onset of flight and arrival of fuels from the fat body is buffered by the high resting concentration of proline. This mechanism seems to have been perfected in the tsetse fly and some dung beetles, where proline metabolism alone can meet the biological demands (Bursell, 1981; Gäde, 1997a,b).

Many scarab beetles are capable of warming-up (see Heinrich, 1993) and it has been shown, for at least a few species, that proline is a major flight substrate (Gäde, 1997a,b; present study). In addition, proline was found to be the sole fuel for

endothermic warm-up in *P. sinuata* (section 4.3.1.). It can therefore be assumed that the use of proline for warm-up is also widespread in beetles.

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