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REVIEW

INSECT FLIGHT MUSCLE METABOLISM

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1. INTRODUCTION

The flight of an insect is of a very complicated and extremely energy-demanding nature. Wingbeat frequency may differ between various species but values up to 1000 Hz have been measured. Consequently metabolic activity may be very high during flight and the transition from rest to flight is accompanied by an increase of 50–100-fold in metabolic rate. Small mammals running at maximal speed and flying birds achieve metabolic rates exceeding resting levels by only 7–14-fold. The exaggerated metabolic rate during insect flight is not accompanied by an oxygen debt, which implies—apart from metabolic adaptations—ample availability of oxygen in the organs responsible for flight (cf. Kammer and Heinrich, 1978). Metabolic rate therefore can be estimated, apart from the depletion of fuel depots, by rates of oxygen consumption. Table 1 shows metabolic rates expressed on this basis for a number of insects from several orders.

Oxygen is carried to the flight muscles by means of tracheae, the fine ramifications of which (tracheoles) invade the flight muscle fibres and come in close apposition with the mitochondria. The intracellular diffusion pathway is thus greatly reduced. Whereas in small insects diffusion of oxygen meets the high metabolic demands, in large insects the primary tracheae and air sacs are ventilated (Weis-Fogh, 1967).

This review is concerned with the main processes in the insect directed to supplying energy for flight. As generally only small amounts of energy-rich substrates are accumulated in the flight muscles themselves, whereas most of them are stored in the fat body, flight metabolism has an impact on various organs, including fat body, haemolymph and flight muscles. Therefore, flight-related phenomena in these organs will be discussed, but prior to this, the main aspects of the mechanism of flight muscle contraction will be considered.

Recent reviews have discussed substrate utilization in flight muscles (Sacktor, 1975; Crabtree and Newsholme, 1975; Steele, 1981), substrate transport (Bailey, 1975; Candy, 1981; Chino, 1981), flight and lipid metabolism (Downer and Matthews, 1976; Beenakkers *et al.*, 1981a, b; Van der Horst, 1983; Beenakkers, 1983), proline and insect flight (Bursell, 1981). General flight metabolism is covered by Kammer and Heinrich (1978) and Beenakkers *et al.* (1984).

2. FLIGHT MUSCLE CONTRACTION AND WINGBEAT

In most species wingbeat is accomplished by the operation of indirect flight muscles, essentially located in the meso- and meta-thoracic segments. Contraction of the dorsal longitudinal flight muscles, extending from prephragma to postphragma, causes depression of the wings, whereas wing elevation is effected by contraction of the dorsoventral flight muscles, inserted on scutum and on sternum or coxa. Contractions cause distortion of the shape of the thorax, which induces the movements of the wings, enabled by a particular articulation between wings and scutum.

In several insect orders (a.o. Orthoptera, Neuroptera, Lepidoptera) wingbeat frequency is determined by the activity of the central nervous system. Each contraction of the flight muscles is induced by a volley of impulses in the motor nerve.

Table 1. Oxygen consumption rates of insects during rest and in flight

Species	$\mu l\ O_2\ g$ body $wt^{-1}\ min^{-1}$ Rest	Flight	Reference
Periplaneta americana (Dictyoptera)	6	600	Polacek and Kubista (1960)
Schistocerca gregaria (Orthoptera)	10	500	Krogh and Weis-Fogh (1951)
Metopsilus procellus (Lepidoptera)	12	1700	Zebe (1954)
Tabanus affinis (Diptera)	18	930	Hocking (1953)
Apis mellifera (Hymenoptera)	53	1000	Hocking (1953)

In these so-called synchronous muscles excitation and contraction are coupled and flight is characterized by wingbeat frequencies not exceeding about 100 Hz. In other orders (Heteroptera, Diptera, most Hymenoptera and Coleoptera) the frequency of muscle contractions exceeds frequency of motor nerve impulses. These asynchronous muscles contract when stretched by the distortion of the thoracic wall. Wings, thorax and flight muscles thus form a resonant system; nerve command only serves to keep the muscles in an active state. The contracting flight muscles produce a small-amplitude deformation of the thorax, causing stretching of the antagonistic muscles. This stretching evokes activation of the muscles, giving rise to contraction. In this way mechanical activity is maintained by a self-oscillatory mechanism, enabling very high wingbeat frequency (for review see Pringle, 1976). The molecular basis of stretch-activation is not fully understood. Stretching by only 1% may evoke substantial activation of muscular ATPase. Even in a demembraned muscle stretch-activation takes place, indicating that it is a property of the contractile protein assembly. The thick filaments of the asynchronous muscle extend to the Z-discs and in the stretched situation material connecting the thick filaments to the Z-disc has been discovered. Particular proteins, thought to function in the connecting process, have been demonstrated in the thick filaments (connecting protein and projectin), in the Z-discs (two hydrophobic proteins), and also in the thin filaments (arthrin). Stretching of the muscle would strain the thick filament, possibly resulting in structural changes, which enhance the interaction between myosin and actin (cf. Bullard, 1983). Although progress has been made in solving the question of mechanical activation, further attention is required on the physical properties of the various connecting proteins, their precise localization and biochemical role in changes of the assembly of the filamentous structures in the muscle.

3. METHODS FOR IDENTIFICATION OF FLIGHT SUBSTRATES

To examine which substrates are actually used during flight a variety of techniques and methods have been developed. Methods with a general applicability will be discussed in this section, whereas more

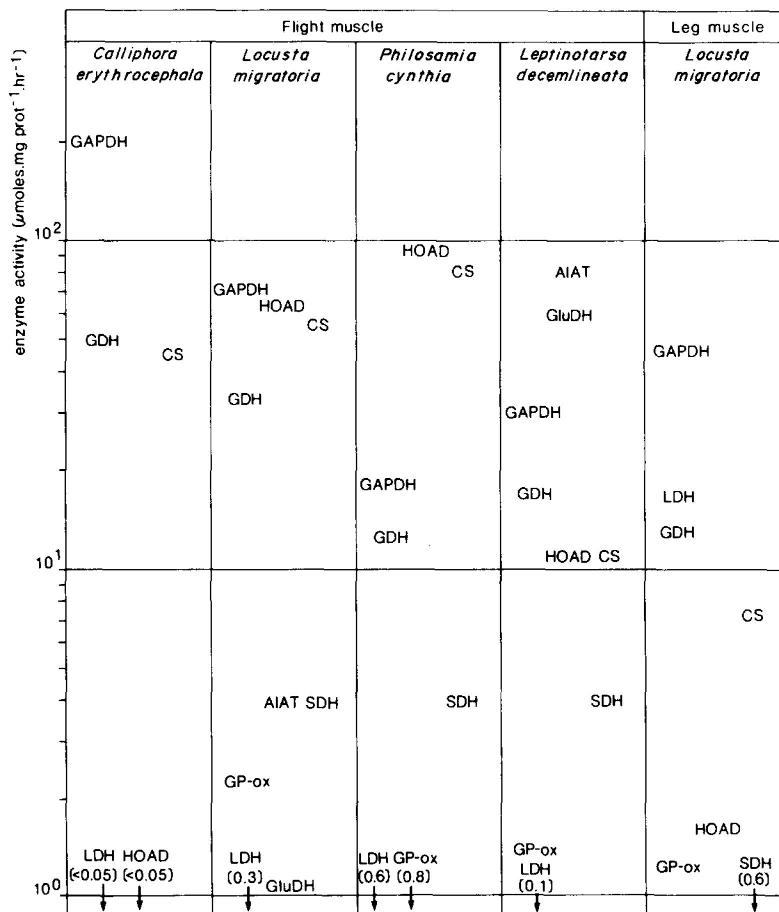

Fig. 1. Enzyme activities in flight muscles of various insects and leg muscle of the locust. Activities are plotted in a logarithmic scale. Data from Beenakkers (1969a), Beenakkers et al. (1975), and Kahn and De Kort (1978). Abbreviations: GAPDH glyceraldehyde-phosphate dehydrogenase; LDH lactate dehydrogenase; GDH glycerol-3-phosphate dehydrogenase; GP-ox glycerol-3-phosphate; FAD oxidoreductase; HOAD 3-hydroxyacyl-CoA dehydrogenase; A1AT alanine aminotransferase; GluDH glutamate dehydrogenase; CS citrate synthase; SDH succinate dehydrogenase.

Table 2. System correlations in insect muscles

Ratio	Flight muscles *Calliphora*	*Locusta*	*Philosamia*	*Leptinotarsa*	Leg muscle *Locusta*
LDH/GAPDH		43.10^{-4}	33.10^{-3}	33.10^{-4}	0.4
GAPDH/GDH	4.0	2.1	1.4	1.8	3.4
CS/SDH		13.9	19.0	2.8	12.2
GDH/GP-ox		14.2	15.8	14.1	10.7
GAPDH/HOAD	$> 10^4$	1.0	0.2	2.7	28.9
AlAT/SDH		1.0		20.0	

Ratios were calculated using enzyme activities depicted in Fig. 1.

specific techniques, confined to detailed aspects of metabolism, will be described at the appropriate places.

3.1 *Measurement of enzyme activities*

The significance of a specific metabolic pathway can be estimated by measuring the activities of enzymes involved. For a great number of enzymes standardized tests have been worked out, yielding maximum catalytic activities.

It has been shown that, irrespective of the tissue examined, activities of enzymes belonging to a particular (part of a) pathway are present in identical proportions (constant proportion groups); thus the activity of one enzyme of such a group can be taken as indicative of the capacity of the entire pathway. For instance glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and 3-hydroxyacyl-CoA dehydrogenase (HOAD) represent glycolytic and β-oxidative pathways, respectively. On the other hand enzyme activities showing no constant relations in different tissues (specific proportions) are indicative of tissue-specific relations between the pathways involved. For instance the activity ratio lactate dehydrogenase (LDH): glyceraldehyde-3-phosphate dehydrogenase may be indicative of the degree of aerobiosis of the tissue. Such ratios represent correlations of systems (Pette, 1965). Figure 1 shows enzyme activity patterns in flight and leg muscles and Table 2 presents system correlations derived from the patterns. At the present time it is sufficient to conclude that contrary to the leg muscles, the flight muscles are strictly aerobic as demonstrated by the LDH/GAPDH-ratio and the high citric acid cycle activity. Moreover the activity ratio GAPDH/HOAD induces the conclusions that (1) *Calliphora* relies heavily if not exclusively on carbohydrate utilization, (2) *Philosamia* is largely dependent on fatty acid oxidation, (3) *Locusta* seems to be capable of utilizing both carbohydrate and lipid during flight. It is also shown that *Leptinotarsa* possesses high activities of enzymes related to amino acid metabolism

Most of the enzymes given in Fig. 1 catalyse reversible reactions, so maximum catalytic activities will be higher than the maximum rate of the pathways involved; this rate is determined by irreversible reactions (cf. Crabtree and Newsholme, 1975 and also section 4.4). Therefore the absolute data provide no quantitative measure of the rate of operation of the pathway. They may be used for qualitative purposes and together with the system correlations they are very reliable in comparative studies on substrate utilization by the muscles.

3.2 *Measurement of the respiratory rate of flight muscle mitochondria*

Isolated muscle mitochondria are incubated in a suitable medium, to which a potential substrate or intermediate is added and the rate of oxygen consumption is measured by the Warburg-technique or, more conveniently, by using an oxygen electrode. The degree of intactness of the mitochondria can be estimated from the respiratory control ratio (rate of oxygen uptake in the presence of ADP, i.e. state 3, to the respiratory rate in its absence, state 4) as well as by the state of coupling of oxidative phosphorylation to electron transport through the respiratory chain (i.e. disappearance of P_i versus oxygen uptake, the P/O-ratio). Table 3 shows the results of such an experiment with isolated mitochondria from flight muscles of the Colorado beetle (Weeda *et al.*, 1980a). Pyruvate, but particularly proline is oxidized at high

Table 3. Substrate utilization by isolated flight muscle mitochondria of the Colorado beetle

Substrate	Rate of oxygen uptake (μg atom min^{-1} mg protein^{-1})
Pyruvate (10)	0.297
Pyruvate (5)	0.277
Pyruvate (10), malate (10)	0.342
Malate (10)	0.028
Citrate (10)	0.010
Oxoglutarate (10)	0.074
Proline (30)	1.189
Proline (1.67)	0.685
Proline (1.67), pyruvate (5)	0.697
Glutamate (5)	0.111
Palmitoyl carnitine (0.2), malate (7)*	0.086

Rates relate to state 3 respiration, final concentration of ADP being 1.6 mM. Respiratory control ratio for pyruvate and proline was high, giving infinite control indices and *P/O* ratio for proline oxidation was 2.8, which is about the theoretical value. From Weeda *et al.* (1980a), except* from Khan and De Kort (1978).

Table 4. The effect of DL-octopamine on substrate oxidation in perfused locust thorax preparation

Substrate	Rate of substrate oxidation (μmol g muscle^{-1} min^{-1}) Control	Octopamine (50 μM)	Significance of increase P
[U-^{14}C]glucose	1.41	2.11	< 0.02
[^{14}C]diacylglycerol plus glucose	0.042	0.062	< 0.02
[U-^{14}C]glucose plus diacylglycerol	0.79	0.99	< 0.001

Glucose concentration in all experiments 0.08 M. The diacylglycerol concentration bound to diacylglyerol-carrying lipoproteins about 30 μmol ml^{-1}. From Candy (1978).

rate in the presence of ATP and P_i, whereas fatty acid oxidation apparently is of minor importance in mitochondrial metabolism.

The method has the disadvantage of being performed *in vitro*. Nevertheless, such experiments have produced important clues to the way mitochondria operate in many insect muscles.

3.3 *Experiments using half thorax preparations*

A perfused locust thoracic muscle preparation suitable for experiments on fuel utilization has been described by Candy (1970). The muscle preparation consists of a half thorax with fat body and gut removed but with muscles, nerves, tracheal system, and cuticle left intact, and inserted into a perfusion chamber so that the muscle side of the preparation is bathed in saline. Oxygen is bubbled into the saline and CO_2 produced is collected. The muscle preparation is stimulated via electrodes. After addition of ^{14}C-labelled substrates to the saline, the rate of $^{14}CO_2$ production is a measure for the rate of substrate utilization. The role of exogenous metabolic regulators can be established by supplying them to the medium. Some results obtained with this method (Candy, 1978) are given in Table 4. Data show that octopamine causes a significant increase in oxidation rate of glucose in locust flight muscles. Glucose oxidation is depressed in the presence of the competitive substrate diacylglycerol. Octopamine increases the oxidation rate of either substrate, when both are present, which may indicate that the amine plays a regulatory role in flight muscle metabolism.

The advantage of the method is of course, that flight muscles are kept intact; however, particularly when performed with asynchronous muscles, the metabolic impact of oscillatory contraction cannot be taken into account.

3.4 *Changes in substrate concentration and turnover in haemolymph*

A flight-induced decrease of the concentration of a given substrate in haemolymph may point to its utilization in flight muscle. It, however, cannot be used to estimate its utilization rate, as it may be replenished from substrate stores. Thus for quantitative purposes substrate turnover has to be determined. Zilversmit (1960) described an isotope method to quantify turnover time of a substrate and, if its

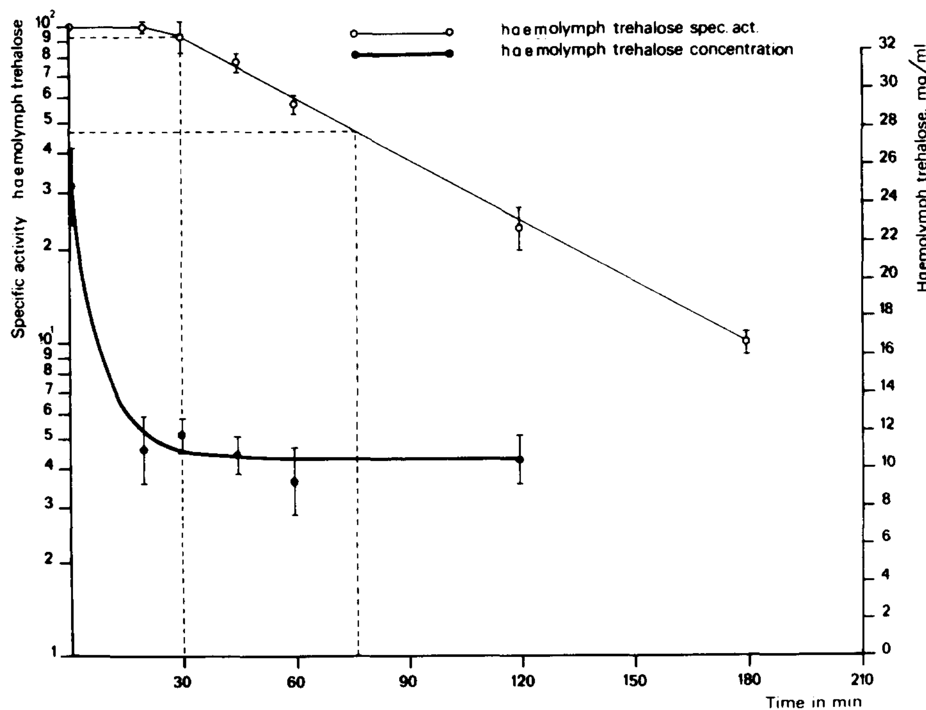

Fig. 2. Changes in the specific radioactivity (plotted semi-logarithmically against time) and the level of the haemolymph trehalose pool in the flying locust. From Van der Horst et al. (1978a).

pool size is known, its utilization rate can be calculated. Figure 2 shows the results of such an experiment by Van der Horst *et al.* (1978a). The haemolymph trehalose pool of migratory locusts was labelled by injection of [^{14}C]trehalose, Locusts were flown and changes in concentration as well as specific radioactivity of trehalose were recorded. During the first half hour of flight, specific activity remains virtually constant, indicating that trehalose is not mobilized from other carbohydrate stores during this period. The decrease in trehalose concentration represents its utilization and, given a haemolymph volume of 250 μl (Beenakkers, 1973), consumption of trehalose is calculated to be 7.3 mg locust^{-1} hr^{-1}. The constant level of haemolymph trehalose reached at 30 min of flight is a steady state, as the decrease of the specific activity demonstrates replenishment of the substrate from non-labelled stores. Turnover time comes to 1.44 × half-time of the specific activity (Zilversmit, 1960), so turnover time of trehalose during the steady state period is 66.2 min. Taking into consideration pool size (10.5 mg ml^{-1}) and haemolymph volume (250 μl) trehalose utilization rate from 30 min of flight is calculated to be 2.4 mg locust^{-1} hr^{-1}.

This *in vivo* method is very reliable, as no interference with normal physiological conduct is introduced. It is also applicable for estimation of substrate turnover in other tissues, provided that a steady state of the substrate exists in the tissue.

4. CARBOHYDRATE AS A FUEL FOR FLIGHT

In most species of Diptera and Hymenoptera carbohydrate constitutes the predominant substrate for flight, whereas in many species of Lepidoptera and Orthoptera this substrate is utilized in combination with lipid (Fig. 1); for example, locusts utilize virtually exclusively carbohydrate at the initiation of flight, whereas lipid is the main fuel during sustained flight, when carbohydrate contributes a minor part to total energy supply.

4.1 *Mobilization of carbohydrate stores*

Insect flight muscles contain a limited amount of carbohydrate reserves, which can meet the energy requirements of the muscles only during the first few minutes of flight. For the maintenance of prolonged flight, carbohydrate from other sources must be mobilized. This exogenous carbohydrate is supplied to the flight muscle via the haemolymph, mainly in the form of trehalose, the principal blood sugar in most insect species (cf. Wyatt, 1967; Bailey, 1975).

Haemolymph trehalose is replenished from glycogen stores in the fat body, the major storage organ of metabolic reserves. Another, though probably less common, carbohydrate reserve for flight activity may be provided by the gut.

In the flight muscles of the blowfly, *Phormia regina*, Sacktor and Wormser-Shavit (1966) found that the trehalose concentration fell rapidly during the first 30 sec of flight, and continued to decrease more slowly thereafter. Initially the glycogen content of the flight muscle did not change, but after 2 min of flight glycogen served as the major fuel until its depletion after about 10 min. In the fat body, glycogen content did not decrease during the first 5 min of flight, but a large depletion was observed after prolonged flights from 15 min. The gut delivers large amounts of dietary glucose into the haemolymph during flight of the blowfly (Hudson, 1958). However, haemolymph glucose remains at a constant (low) level, indicating a rapid turnover during flight. Clegg and Evans (1961) showed that glucose is removed from the haemolymph during flight mainly by its direct conversion into trehalose in the fat body. Thus, in *Phormia* haemolymph trehalose is replenished from fat body glycogen as well as from gut sugars, although the fat body appeared to be the exclusive site for the synthesis of trehalose.

During flight of *Locusta migratoria* the major changes in the carbohydrate reserves of the flight muscles take place during the first 10 min of flight, after which they remain nearly constant at about half the resting values (Worm and Beenakkers, 1980). In *Schistocerca gregaria*, no more than one-third of the flight muscle glycogen was left after a flight of only 10 sec; after 3 min flight no further decrease took place (Rowan and Newsholme, 1979). Thus in the locust flight muscle glycogen is utilized directly at the onset of flight, whereas in blowfly muscle trehalose is the initial substrate, and glycogen is utilized after the first flight minutes.

In *Locusta*, haemolymph trehalose is the major energy source during the first 20–30 min of flight, its concentration decreasing to reach a steady state at about 35% of the resting level after 30 min, as shown previously (Fig. 2). The turnover rate of haemolymph trehalose during the steady state is much lower than in the first flight period, and although lipid has become the major substrate for flight, trehalose still provides about 25% of total energy supply (Van der Horst *et al.*, 1978a). The steady state of haemolymph trehalose is sustained by the mobilization of other carbohydrate stores. Fat body glycogen is the main source, its concentration decreasing at a rate sufficient to account for almost two-thirds of the total trehalose utilized between 30 and 120 min of flight (Van Marrewijk *et al.*, 1980). Thus one-third must be derived from other sources, part of which will be provided by the glycerol that is liberated by hydrolysis of diacylglycerol, as will be discussed in section 5.2. Glycogen from the gut wall seems to be of minor importance for additional trehalose synthesis (Mayer and Candy, 1969a).

4.2 *Trehalose synthesis in fat body*

The pathway for trehalose biosynthesis in insect fat body has been elucidated for *Schistocerca* by Candy and Kilby (1961), who demonstrated that trehalose is synthesized from UDP-glucose and glucose-6-P as shown in Fig. 3, the catalysing enzymes being trehalose-6-P synthase and trehalose-6-phosphatase. The synthesis of glycogen requires UDP-glucose as well, whereas glucose-6-P is an activator of glycogen synthase, which suggests a regulatory system directing the synthetic process to either trehalose or glycogen. This has been investigated by Murphy and Wyatt (1965) using fat body of the silkworm *Hyalophora cecropia*. Trehalose-6-P synthase and glycogen synthase displayed K_m-values for UDP-glucose of respectively 0.3 and 1.6 mM and the former en-

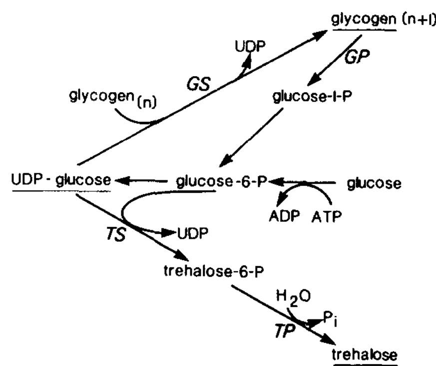

Fig. 3. A schematic representation of the interactions of trehalose synthesis, glycogen synthesis and glycogen breakdown in insect fat body. GS glycogen synthase; GP glycogen phosphorylase; TS trehalose-6-P synthase; TP trehalose-6-P phosphatase.

zyme is inhibited by high trehalose concentrations. Thus, when the trehalose concentration is high and its synthesis inhibited, the UDP-glucose level will rise and permit an increased synthesis of glycogen. When, for example during flight, trehalose concentration decreases, inhibition of trehalose-6-P synthase is revealed and its greater affinity for UDP-glucose allows a preferential synthesis of trehalose. This would also reduce the level of glucose-6-P and thus releave the inhibiting effect of this intermediate on glycogen phosphorylase as found in locusts (Applebaum and Schlesinger, 1973), and glycogen may even be used now for trehalose synthesis (Fig. 3).

4.3 *Hormonal control of trehalose mobilization*

Control by metabolic hormones constitutes another level of regulation of trehalose mobilization. Hypertrehalosemic hormones causing an elevation of haemolymph trehalose levels by stimulating the conversion of fat body glycogen to trehalose, have been demonstrated in various insect species (cf. Candy, 1981; Beenakkers *et al.*, 1984). The presence of an hypertrehalosemic hormone in the corpora cardiaca does not necessarily imply that the insect concerned will exhibit hypertrehalosemia when injected with an extract of corpora cardiaca. For example, corpora

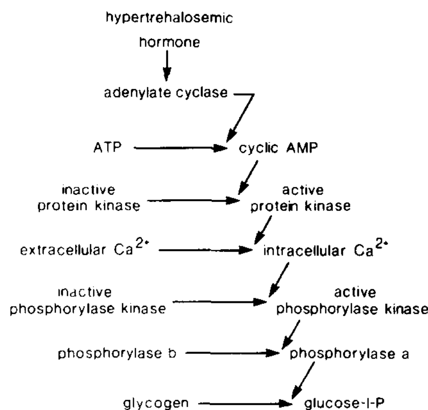

Fig. 4. Scheme showing the possible mode of action of hypertrehalosemic hormone on glycogenolysis in cockroach fat body. After Steele (1982).

cardiaca extracts of *Locusta* induce hypertrehalosemia in *Periplaneta americana*, not however in the locust itself (Chalaye, 1969).

A physiological role of the hypertrehalosemic hormone during flight has been demonstrated in *Calliphora erythrocephala* (Vejbjerg and Normann, 1974). Normal blowflies are able to maintain a constant level of trehalose in their haemolymph during a 45 min flight. After removal or denervation of the corpora cardiaca the trehalose concentration drops markedly during flight and flies lose their ability to fly. When the glands of such flown denervated flies are crushed "*in situ*", trehalose levels increase rapidly to normal, and flight ability is restored. In locusts, Mordue and Goldsworthy (1969) found two hypertrehalosemic factors, one in the storage and the other in the glandular lobes of the corpora cardiaca, the latter being at least twice as potent as the former. Locust corpora cardiaca also contain two adipokinetic peptide hormones: the major factor AKH I, present in the glandular lobe (Goldsworthy *et al.*, 1972), and the minor factor AKH II, located mainly in the glandular, but also present in the storage lobe (Carlsen *et al.*, 1979). Upon injection, both hormones raise diacylglycerol levels in locust haemolymph. Although neither AKH I nor AKH II increase carbohydrate levels in haemolymph of the locust, both hormones elicit hyperytrehalosemia in the cockroach (Jones *et al.*, 1977; Van Norstrand *et al.*, 1980). Jones *et al.*, (1977) concluded that the hypertrehalosemic effect of the locust glandular lobes is due solely to AKH I. A hypertrehalosemic response, though absent in normal locusts, was found however in neck-ligated locusts injected with glandular lobe extract and with purified AKH II, whereas synthetic or extracted AKH I failed to induce this effect (Loughton and Orchard, 1981). The presence of a hypotrehalosemic factor in the endocrine system was suggested to explain the absence of a hypertrehalosemic response in non-ligated locusts. Recently Orchard and Lange (1983) demonstrated the presence of small amounts of both AKH I and II in the haemolymph of resting locusts,and their release from the corpora cardiaca during a 30 min flight.

Hypertrehalosemic hormones stimulate glycogen breakdown in the fat body through the activation of glycogen phosphorylase (cf. Steele, 1982). This enzyme may have two interconvertible forms, active phosphorylase *a* and inactive phosphorylase *b*, the *b* to *a* conversion being catalysed by phosphorylase kinase.

Injection of corpora cardiaca extracts may cause a strong activation of phosphorylase, as has been observed in *Manduca sexta* (Ziegler, 1979) and *Locusta* (Van Marrewijk and Beenakkers, 1979). The activating effect of locust corpora cardiaca is almost entirely due to AKH I (Van Marrewijk *et al.*, 1983). Activation of locust fat body phosphorylase by AKH I, also shown by Gäde (1981), seems to contradict the hypertrehalosemic response observed by Loughton and Orchard (1981) in ligated locusts only after injection of AKH II, AKH I being without effect. The reason for this discrepancy is unknown.

Locust flight also induces a rapid activation of fat body phosphorylase, the active form increasing from less than 10% of total phosphorylase in resting

locusts to about 30% during sustained flight (Van Marrewijk *et al.*, 1980).

The mode of action of the hypertrehalosemic hormone in the fat body has been investigated extensively in *Periplaneta*. By activating adenylate cyclase, the hormone induces a rise in the level of cAMP in the fat body *in vivo*, while *in vitro* protein kinase activation in cockroach fat body extracts was demonstrated after addition of the hormone as well as cAMP (Hanaoka and Takahashi, 1977, 1978). McClure and Steele (1981) showed that both cAMP and Ca^{2+} are obligatory participants in hormone-induced phosphorylase activation, and a model was presented in which hormone-activated protein kinase regulates the permeability of the cell membrane, giving rise to increased intracellular Ca^{2+}-levels; then Ca^{2+} in turn activates phosphorylase kinase (Fig. 4).

In *Locusta*, flight activity as well as corpora cardiaca extracts elevate the level of cAMP in fat body (Gäde and Holwerda, 1976), which also contains cyclic nucleotide-dependent protein kinase (Pines and Applebaum, 1978; Van Marrewijk *et al.*, 1980). However, the actual mechanism of phosphorylase activation by adipokinetic hormone still remains to be elucidated.

4.4 *Utilization of carbohydrate in flight muscles*

Flight muscle glycogen may constitute an important source of energy during flight. In *Periplaneta* at least 85% of all carbohydrate used during flight is derived from flight muscle glycogen (Downer and Matthews, 1976). The mechanism for controlling the glycogenolytic rate induced on the initiation of flight in blowfly flight muscles has been studied by Childress and Sacktor (1970). They showed that phosphorylase *b* is virtually inactive because of its high K_m for AMP and P_i compared to the *in vivo* levels of both ligands, and by the inhibitory action of ATP. In contrast phosphorylase *a*, being independent of AMP levels and not inhibited by ATP, has a K_m for P_i which approximates the P_i concentration in the muscle. Thus the conversion of phosphorylase *b* to *a* controls the rate of glycogenolysis. It was calculated that to meet the high glycogenolytic rate during flight, at least 50% of the total phosphorylase must be in the *a* form. Indeed, the level of phosphorylase *a* increased from 18% at rest to 72% after 15 sec of flight.

Phosphorylase kinase was shown to be activated not by protein kinase as occurs in vertebrate skeletal muscle, but by Ca^{2+} (Sacktor *et al.*, 1974). Since muscle contraction also requires Ca^{2+}, the Ca^{2+}-activation of phosphorylase kinase was suggested to be essential in the mechanism coupling contraction to glycogenolysis.

Utilization of trehalose, the major form in which exogenous carbohydrate is supplied to the flight muscles, requires its hydrolytic cleavage into two glucose residues, a reaction catalysed by trehalase. Both soluble and particulate trehalases in the flight muscles have been described. Several authors postulated that the soluble enzyme is of haemolymph origin (Clements *et al.*, 1970; Friedman and Alexander, 1971), but Duve (1975) showed that depending on the isolation medium used, part of the particulate trehalase may be recovered in the soluble fraction. So, particulate trehalase will constitute the major part, if not all, of the enzyme. The activity in the particulate fraction has been shown to reside in the mitochondria of *Phormia*, *Sarcophaga*, *Calliphora* (cf. Duve, 1975) and *Apis mellifera* (Brandt and Huber, 1979), whereas it occurs in the microsomal fraction of *Hyalophora*, *Blaberus* and *Locusta* (cf. Worm, 1981). These different localizations may reflect a difference between insects that use only carbohydrate and insects that use mainly lipids as a fuel for flight (Duve, 1975). The latter insects generally have synchronous muscles, characterized by a well-developed sarcoplasmic reticulum, whereas the carbohydrate utilizing insects possess asynchronous muscles, with markedly reduced sarcoplasmic reticulum.

Trehalose hydrolysis has been suggested to be a control point in blowfly (Sacktor and Wormser-Shavit, 1966) and locust (Ford and Candy, 1972) flight muscles, but the control mechanism of trehalase activity is still unknown.

Neither possible physiological effectors as Ca^{2+}, octopamine, or adipokinetic hormone, nor dibutyryl-cAMP had any effect on trehalase activity in locust flight muscle (Worm, 1981). The enhancement of trehalose oxidation in locust muscles by octopamine (Candy, 1978) may indicate stimulation of glycolytic flux rather than trehalase activation, since octopamine stimulates glucose oxidation as well (Table 4).

Both glycogen and trehalose breakdown lead to the formation of glucose-6-P, catabolism of which up to pyruvate in flight muscles is in essence identical with vertebrate glycolysis. In contrast to most other muscle types, flight muscles exhibit complete oxidation of pyruvate, lactate dehydrogenase being virtually absent (Fig. 1). Reoxidation of cytoplasmic NADH, derived from the oxidation of glyceraldehyde-3-P, is accomplished by the conversion of a catalytic amount of dihydroxyacetone-P to glycerol-3-P, catalysed by the cytoplasmic glycerol-3-P dehydrogenase. Glycerol-3-P enters the mitochondrion and is reoxidized by the mitochondrial glycerol-3-P oxidase, transferring the hydrogen to the respiratory chain. The regenerated dihydroxyacetone-P is then available for further oxidation of cytoplasmic NADH. This glycerol-3-P shuttle ensures that glycolysis continues aerobically, converting all glycosyl units into pyruvate, to be oxidized in the mitochondria (cf. Sacktor, 1975). In most flight muscles the capacity of the shuttle meets the glycolytic capacity, and thus is not rate-limiting for the metabolism of glucose (Crabtree and Newsholme, 1972a, 1975). Crabtree and Newsholme (1972a) also demonstrated that the *in vitro* activities of the regulatory glyco(geno)lytic enzymes phosphorylase, hexokinase, and phosphofructokinase agree well with rates of carbohydrate utilization calculated from oxygen uptake data during flight (Table 5). These activities thus may indicate utilization rates of carbohydrate during flight.

Since in *Schistocerca* flight muscle glycogen reserves are depleted already after a 10 sec flight (Rowan and Newsholme, 1979), hexokinase, essential for breakdown of exogenous glucose, must quickly become maximally active to meet the calculated glycolytic flux rate (Table 5). However, glucose-6-P, whose concentration increases during the first minutes of flight (Worm and Beenakkers, 1980), is a

Table 5. Activities of some regulatory enzymes of glyco(geno)lysis and rate of carbohydrate utilization in various flight muscles

Insect species	Enzyme activities (μmol min^{-1} g fresh wt^{-1}) Phosphorylase	Hexokinase	Phosphofructokinase	Rate of carbohydrate utilization*
Schistocerca gregaria	7.5	11.5	17.0	14.0
Periplaneta americana	30.0	18.0	19.0	15.0
Apis mellifera	4.0	29.0	20.0	32.0
Calliphora erythrocephala	55.0	35.0	43.0	59.0†

*Utilization rate (μmol of C_6 unit min^{-1} g fresh wt^{-1}) calculated from respiratory rates obtained on flying insects when carbohydrate was the main energy source. Oxygen uptake is attributed to flight muscle activity. †Value from related species, *Lucilia sericata*. From Crabtree and Newsholme (1972a).

potent inhibitor of hexokinase. Storey (1980a) has demonstrated that despite strongly inhibitory levels of glucose-6-P, during flight the inhibition is reversed by the additive effects of P_i and L-alanine, P_i being liberated from the breakdown of arginine-P, and alanine accumulating until the citric acid cycle is fully activated.

The involvement of glucose-6-phosphatase in the regulation of glucose metabolism in muscle has been proposed by Surholt and Newsholme (1981). The strong positive correlation between the activities of hexokinase and glucose-6-phosphatase in various muscles from different animals suggests that the role of the phosphatase is to produce a substrate cycle between glucose and glucose-6-P (Fig. 5). This cycle was shown to exist indeed in the hawk moth, *Acherontia atropos* (Surholt and Newsholme, 1983). In the flight muscle, the rate of cycling was low at rest but increased about 65-fold during flight. The increase in the cycling rate during flight may enhance the sensitivity of the rate of glucose phosphorylation to changes in concentrations of effectors of hexokinase. Thus a higher glycolytic rate may be achieved under conditions that require a higher power output, for example an increased speed of flight, or an increase of wind resistance.

In the flight muscles of bumblebees, *Bombus* spp., a substrate cycle between fructose-6-P and fructose-1,6-diP has been proposed previously by Newsholme *et al.* (1972) (Fig. 5). Bumblebees are unable to fly when their thoracic temperature is less than 30°C. During flight, heat generated by muscle contraction keeps thoracic temperature sufficiently high; for

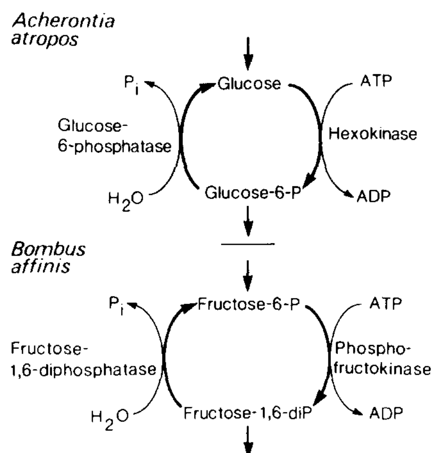

Fig. 5. Substrate cycles in the flight muscles of the hawk moth, *Acherontia atropos*, and the bumblebee, *Bombus affinis*.

periods of rest or food collection it was suggested that thoracic temperature is maintained at a level suitable for flight by the generation of heat derived from the hydrolysis of ATP via substrate cycling. This heat generating role of the cycle was confirmed for *Bombus affinis* by Clark *et al.* (1973). In flying bumblebees, no substrate cycling was found, whereas in the non-flying condition the cycling rate was much higher at an ambient temperature of 5 than at 21°C (Table 6). Thus in contrast to the substrate cycle in *Acherontia* which is active during flight, the cycle in *Bombus* operates in the non-flying insect and is inactive during flight, the difference emphasizing the different roles of the two cycles.

5. LIPID AS A SUBSTRATE FOR FLIGHT

Particularly insects performing long range flights (e.g. Lepidoptera and Orthoptera) rely heavily on lipid as a fuel for flight muscle metabolism because of its high energy yield. Although after take off carbohydrate consumption may prevail, progressive fatty acid oxidation in the flight muscles results in a principally lipid-fuelled metabolism when flight is sustained.

In general, lipid reserves in the flight muscles themselves are very limited, so during flight lipid reserves accumulated in the fat body have to be mobilized, transported in the haemolymph, and taken up by the flight muscles.

5.1 *Haemolymph lipid*

The lipid content in the haemolymph may vary considerably amongst insect species (cf. Bailey, 1975); however, in all but a few species examined thus far, diacylglycerol (DG) constitutes the dominant neutral lipid class in the haemolymph (cf. Gilbert and Chino, 1974; Downer and Matthews, 1976; Beenakkers *et al.*, 1981a, b, 1984).

The insect fat body is the metabolically most important organ in the supply of lipid to the haemolymph during exercise. Tietz (1962, 1967) demonstrated lipid release from adult *Locusta* fat body. *In vitro* incubation of fat body in a medium containing ^{14}C-labelled fatty acids led to incorporation of the radiolabel into acylglycerols, and upon transfer of the prelabelled fat body into a medium obligatory containing haemolymph, acylglycerol was released, Chino and Gilbert (1964), working on prelabelled fat bodies of *Hyalophora* and *Melanoplus differentialis* were the first to demonstrate the specific release of DG.

Flight activity of the locust is accompanied by a marked elevation of the haemolymph DG level even-

Table 6. Effect of environmental temperature and flight on the cycling rate between fructose-6-P and fructose-1,6-diP and on the rate of glycolysis in the bumblebee

	Environmental temperature			
	21°C		5°C	
Rate of	Flying	Non-flying	Flying	Non-flying
Fructose-6-P phosphorylation	14.7	1.6	20.4	16.2
Substrate cycling	0	0.48	0	10.4
Glycolysis	14.7	1.12	20.4	5.8

Rates are expressed in μmol min^{-1} g fresh wt^{-1}. From Clark *et al.* (1973).

tually reaching a plateau value at about 3-fold its resting concentration (Mayer and Candy, 1969a; Beenakkers, 1973; Jutsum and Goldsworthy, 1976). Direct indications for the actual use of DG for flight muscle metabolism were provided by Van Handel and Nayer (1972) in the moth *Spodoptera frugiperda*, as both during rest and flight the turnover of the pools of DG appeared to be sufficiently high to match the metabolic activity of the insects. These data were inferred from homogenates of total insects, thus revealing no evidence for a transport of lipids. In *Locusta*, turnover of the DG pool in the haemolymph has been studied using pulse-labelling techniques (Van der Horst *et al.*, 1978b). Insects flown for 2 hr were injected with [^{14}C]oleic acid, yielding radiolabelled DG with a high specific radioactivity whereas incorporation of radiolabel into fat body triacylglycerols (TG) was negligibly low. Upon continuation of flight, specific radioactivity diminished logarithmically with time (Fig. 6), indicating that the constant DG level conceals a steady state in which utilization of haemolymph DG is matched by replenishment from non-labelled fat body TG. Turnover rate during flight is accelerated more than 8-fold over the resting value and amounted to 3.4 mg DG $locust^{-1}$ hr^{-1}. Based on oxygen consumption, Beenakkers (1965) calculated a lipid utilization of 4.1 mg hr^{-1} if lipid was the sole respiratory fuel. Considering the participation of trehalose during longer flight as well, (section 4.1), this calculated value is in fair agreement with the actual energy consumption.

Oxidation of DG in the first period of flight is increasing concomitant with the rise in haemolymph DG level. as was demonstrated by measuring $^{14}CO_2$ production by flying locusts of which the haemolymph DG pool had been prelabelled with [^{14}C]oleic acid in the resting situation (Van der Horst *et al.*, 1980) (Fig. 7). Apparently, lipid oxidation reaches a plateau value between 30 and 45 min after the initiation of flight. The oxidation rate thus determined for the period of 45 to 60 min of flight is in close agreement with the data obtained from the above turnover experiments. As, however, tethered flight was applied, values may be significantly higher under natural conditions.

Concerning the structure of the haemolymph DG, Tietz *et al.* (1975) showed that 1,2-DG are released from locust fat body. These 1,2-DG are stereospecific, revealing the *sn*-1,2 configuration with a remarkably high purity (Tietz and Weintraub, 1980; Lok and Van der Horst, 1980). The former authors analysed fat body and haemolymph 1,2-DG of locusts at rest, using enantioselective enzymatic degradations, whereas the latter authors applied a ^{1}H-NMR method using chiral shift reagents after chemical modification of the haemolymph 1,2-DG obtained from locusts at rest and after flight, evidencing stereospecificity of the processes involved in the production of these DG in the fat body.

5.2 *Lipid mobilization from fat body*

The initial event in the utilization of fat body lipid as a fuel for flight is the hydrolysis of TG by the action of lipases (cf. Van der Horst, 1982).

In mammalian adipose tissue, mobilization of TG is a hormone-controlled reaction catalysed by the intracellular "hormone sensitive lipase", which may actually encompass a number of enzymes specific for tri, di and monoacylglycerols. The enzyme system can be activated by a wide array of lipolytic hormones, the most likely activation pathway being adenylate cyclase stimulation, intracellular cAMP accumulation, activation of a cAMP-dependent protein ki-

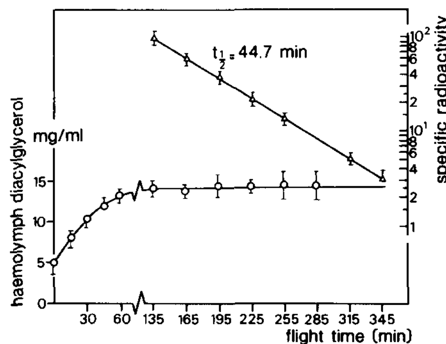

Fig. 6. Decrease in specific radioactivity of the haemolymph diacylglycerol pool in flying locusts, labelled with [^{14}C]oleic acid at the steady state level. After Van der Horst *et al.* (1978b).

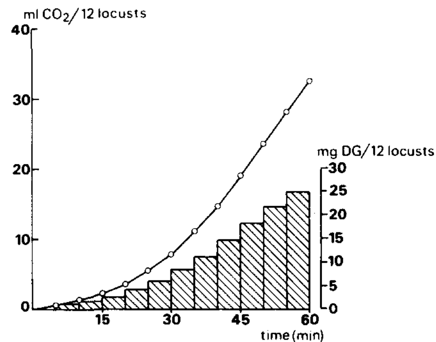

Fig. 7. Carbon dioxide production from a group of 12 locusts due to diacylglycerol fatty acid oxidation during flight. Calculations are based both on $^{14}CO_2$ production from, and changes in specific radioactivity of diacylglycerols, pulse-labelled with [^{14}C]oleic acid. From Van der Horst *et al.* (1980).

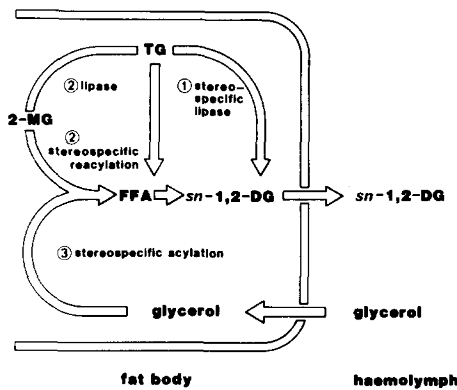

Fig. 8. Alternative routes for *sn*-1,2-diacylglycerol production in locust fat body.

nase followed by phosphorylation and activation of the lipase (cf. Steinberg, 1976). In the locust, particularly AKH I functions in relation to flight activity; injection of the hormone into resting locusts evokes a rapid elevation of haemolymph DG (Beenakkers, 1969b; Mayer and Candy, 1969b) at the expense of fat body TG (cf. Mordue and Stone, 1979). As already discussed in section 4.3, the hormone, as well as flight activity, induces an increase in fat body cAMP, and this nucleotide activates *in vitro* protein kinase (cf. Beenakers *et al.*, 1978). Thus, apart from its activating effect on glycogen phosphorylase, the hormone would seem the most likely candidate for fat body lipase activation. Pines *et al.* (1981) accomplished an *in vitro* stimulation of locust fat body TG lipase by cyclic nucleotides. Despite these *in vitro* results, hormone-dependent activation of neither protein kinase nor lipase could be demonstrated *in vivo*, leaving the ultimate mechanism underlying hormonal control of TG mobilization to be resolved.

As to the production of DG from TG in the insect fat body during flight, two possible mechanisms have been proposed. Spencer and Candy (1976) suggested monoacyl cleavage of the stored TG to be the primary route of 1,2-DG production (Fig. 8). This pathway may prevail in the fat body of the cockroach (Hoffman and Downer, 1979). An alternative is degradation of TG to 2-monoacylglycerol (2-MG) followed by a reacylation to 1,2-DG (Tietz and Weintraub, 1978, 1980). The observed *sn*-1,2 configuration of the DG in locust haemolymph and fat body favours the latter mechanism (Fig. 8). Tietz *et al.* (1975) showed that fat body microsomal acyltransferase specifically used 2-MG; moreover, the enzyme was stereospecific and preferentially synthesized *sn*-1,2-DG (Tietz and Weintraub, 1980). Lok and Van der Horst (1980) studied the positional distribution of the fatty acids of *Locusta* fat body TG and haemolymph *sn*-1,2-DG; the minor difference between the *sn*-1 position of the DG and the primary positions of the TG are compatible with the 2-MG pathway, although direct production of *sn*-1,2-DG by a stereospecific lipase cannot be ruled out either. Incubation experiments with the fat body lipase system and optically active TG labelled at the primary positions of glycerol could perhaps distinguish more conclusively between the possibilities.

In fact, in the locust, the problem is even more complicated. Candy *et al.* (1976) showed in the desert locust that injection of [^{14}C]glycerol resulted in the appearance of both labelled trehalose and DG in the haemolymph, and they proposed that when lipid is used by the flight muscles, most of the remaining glycerol is transported to the fat body and converted to trehalose and DG. In the migratory locust, this concept has been substantiated and extended (Van der Horst *et al.*, 1983); using pulse-labelling of the haemolymph glycerol pool during flight a rapid transport of glycerol from the flight muscles to the fat body was shown. In view of the exclusive stereospecificity of the DG recovered in the haemolymph during flight the production of DG from fatty acids and glycerol in the fat body obviously also is a stereospecific process, involving stereospecific acylation (Fig. 8).

The reason for these stereospecific processes is not known. Tentatively, Van der Horst (1983) related chiral DG to its use by a stereospecific flight muscle (lipoprotein) lipase, since in the cockroach, the one other insect in which stereospecificity of haemolymph DG has been investigated, nearly equal amounts of both enantiomers were found (Hoffman and Downer, 1979) and as indicated before, in this insect flight is powered by carbohydrate and no DG is used.

5.3 *Lipid transport*

As indicated above, *in vitro* release of DG from insect fat body requires the presence of haemolymph components in the incubation medium, attributable to specific lipoproteins in the haemolymph which function in taking up and carrying the lipid in the aqueous medium. Haemolymph lipoproteins have been identified in several insect species, in particular silkmoths, locusts, and cockroaches (cf. Wyatt and Pan, 1978). In Lepidoptera, two major DG-transporting lipoproteins were identified in *Hyalophora cecropia* and *H. gloveri* (Thomas and Gilbert, 1968, 1969), and in *Philosamia cynthia* (Chino *et al.*, 1969). Both lipoproteins (named Lp-I and LP-II) are high-density lipoproteins with high molecular weights [approx. 700,000 (LP-I) and 500,000(LP-II)]. LP-I accepts DG from the fat body and carries them to other tissues, which may include the flight muscles. LP-II appeared to be the female-specific vitellogenin (Chino *et al.*, 1976). Since in a number of insect species the sex-unspecific LP-I serves to transport cholesterol and hydrocarbons in addition to DG (Chino and Gilbert, 1971; Chino and Kitazawa, 1981). Chino *et al.* (1981) proposed the term "lipophorin" for these haemolymph lipoproteins.

At present, the dynamics of loading of DG-carrying lipoproteins (or lipophorins) during flight have been described only for locusts. The lipophorins are synthesized in the fat body (Harry *et al.*, 1979; Gelissen and Wyatt, 1981); inhibition of protein synthesis *in vitro*, however, does not affect the release of DG nor the uptake of lipid by lipoproteins, indicating that the lipoproteins can be loaded with lipid independently of *de novo* synthesis (Peled and Tietz, 1973).

Application of gel filtration chromatography techniques has resulted in the isolation and partial characterization of lipoprotein fractions from the haemolymph of *Locusta*. In resting locusts a yellow

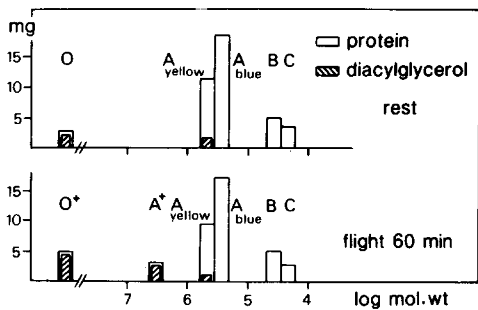

Fig. 9. Elution profiles of locust haemolymph (lipo)proteins, isolated by gel filtration chromatography on Ultrogel AcA 22 of haemolymph (1.0 ml) from locusts at rest and after a 60 min flight. Fractions were assayed for protein and DG content. Essentially after Van der Horst *et al.* (1979).

lipoprotein fraction (A_{yellow}), mol.wt approx. 450,000, and a very high mol. wt lipoprotein fraction (O), recovered in the void volume of the column carry the haemolymph DG. In addition, three non-lipid containing protein fractions (A_{blue}, B, and C) of lower mol. wt are present (Fig. 9). Increased lipid mobilization due to flight activity or injection of AKH was attended by a depletion of both lipoprotein A_{yellow} and protein fraction C, whereas a new high lipid loaded lipoprotein (A^+) (mol. wt about 3,500,000) appeared along with an increase in amount and lipid content of the lipoprotein fraction O (O^+) (Mwangi and Goldsworthy, 1977, 1981; Van der Horst *et al.*, 1979) (Fig. 9). These changes in the haemolymph lipoproteins during flight, resulting in a higher capacity for DG uptake and transport in the haemolymph, are essential for the progressive turnover rate of DG during flight (Van der Horst *et al.*, 1979; Van der Horst and Beenakkers, 1980). Interestingly, unlike lepidopteran LP-I, which is a high-density lipoprotein, A^+ apparently is a low-density lipoprotein resembling human low-density lipoproteins (Goldsworthy and Wheeler, 1982).

Since total protein concentration in the haemolymph remains unaffected during flight or after AKH injection, the changes in the lipoprotein fractions apparently originate from reorganizations of proteins already present in the haemolymph in the resting stage. Immunological evidence pointed to an involvement of the apoprotein from A_{yellow} and protein fraction C in the formation of flight-specific lipoproteins (Van der Horst *et al.*, 1981a), as also suggested by Mwangi and Goldsworthy (1977, 1981). Experiments in which isolated and radiolabelled lipoprotein and protein fractions were reinjected into locusts and dynamics followed have convincingly demonstrated this protein rearrangement (Van der Horst *et al.*, 1981b; Wheeler and Goldsworthy, 1983a, b).

The increased amount of DG carried by lipoprotein fractions A^+ and O^+ during flight is principally derived from the enhanced release of lipids from the fat body, though transfer of DG from A_{yellow} also takes place (Van der Horst *et al.*, 1981b). Lipoprotein A^+ apparently is the transport protein for the DG supplying energy to the flight muscles, as will be discussed in the following section. The function of the chylomicron-like fraction O (or O^+) currently remains unclear.

The changes in lipoprotein reorganizations and lipid loading in the flying insect allow efficient delivery of lipid to the contracting flight muscles without degradation of the transport proteins, which is reflected by the structural features of insect lipoproteins (Pattnaik *et al.*, 1979; Van der Horst, 1982, 1983). An intriguing question remains why protein fraction C participates in the lipoprotein fractions elevated during flight. A few explanations have been advanced (cf. Van der Horst, 1983; Beenakkers *et al.*, 1984), including activation of the flight muscle (lipoprotein) lipase in a way analogous to the activation of mammalian lipoprotein lipase by apoproteins (cf. Cryer, 1981; Sparrow and Gotto, 1982). In this respect it is of interest that only one specific protein of the C-fraction binds to lipoprotein A_{yellow} during A^+ formation (Van der Horst, Van Doorn and Beenakkers, in preparation). This C-protein is a glycoprotein with a rather complex sugar composition, and also the lipoprotein fractions contain carbohydrate moieties. It is feasible that one of the functions of the sugars included in mammalian proteins, namely, determinants of protein recognition by its target cell (cf. Olden *et al.*, 1982), may apply to the locust as well.

5.4 *Lipid utilization in flight muscles*

The mechanisms by which haemolymph lipids enter the flight muscles are virtually unknown. Fatty acids from haemolymph acylglycerols are made available for oxidation in flight muscle mitochondria by the hydrolytic action of flight muscle (lipoprotein) lipases. In addition, extracellular hydrolysis by haemolymph lipases as found in the hawk moth *Deilephila* (Chang, 1977) and the cockroach (Downer and Steele, 1973; Hoffman and Downer, 1979) may be important as well. Flight muscle lipase of *Hyalophora cecropia* hydrolysed DG at a relatively high rate, suggesting that DG is separated from its carrier lipoprotein before entering the muscles (Gilbert *et al.*, 1965). In view of the increased level of fatty acids in locust haemolymph during flight, the possible extracellular hydrolysis of DG prior to uptake is discussed by Downer (1978). Lipolytic activity in flight muscles has been reported for many insect species (Crabtree and Newsholme, 1972b; Male and Storey, 1981), The high DG lipase activity in locust, waterbug, dragonfly and some moths is consistent with the role of this lipid class as the main fuel for flight in these insects.

In the locust, particularly lipoprotein A^+ functions in supplying DG for the generation of energy to the flight muscles. Using this lipoprotein as a substrate, Robinson and Goldsworthy (1977a) showed a strong increase in DG utilization rate by locust flight muscles in a half thorax preparation upon supply of corpus cardiacum extract, whereas utilization of DG bound to lipoprotein A_{yellow} was low and not influenced by the hormone. Van der Horst, Storm, Van Doorn and Beenakkers (in preparation), following conversions of ^{125}I-labelled lipoproteins injected into flying locusts, have also shown that DG carried specifically by A^+ are utilized by the flight muscles.

Recently, in the locust the first direct evidence was established that flight muscle homogenates are capable of hydrolysing lipoprotein-bound acylglycerols, and the enzymes responsible may therefore be re-

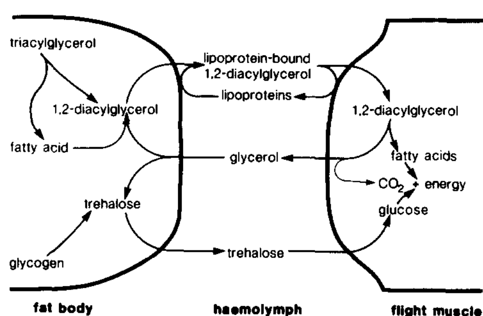

Fig. 10. Survey of substrate cycles involved in glycerol conversions during locust flight. From Van der Horst *et al.* (1983).

garded as lipoprotein lipases (Wheeler *et al.*, 1984). A radiochemical assay was used in which neutral lipids, present as part of haemolymph lipoproteins (principally lipoprotein A^+), served as substrates. The radio-label was located in the glycerol backbone of DG, and lipolytic activity in the flight muscles was measured by the release of the labelled glycerol (see below). The activities measured are well in excess of the DG utilization rate estimated in flight as previously discussed. The possible role of locust lipoprotein apoproteins, particularly protein C, in the activation of the lipoprotein lipase is under investigation. As the locust haemolymph 1,2-DG is stereospecific (as indicated before), the possible stereospecificity of the lipoprotein lipases might be anticipated (cf. Jensen *et al.*, 1983).

Glycerol produced in the flight muscles by the lipolysis of acylglycerols may be converted to glycerol-3-phosphate and metabolized further. However, isolated locust flight muscle is only capable of oxidizing glycerol at low rates (Candy, 1970; Robinson and Goldsworthy, 1977a) and activity of glycerol kinase is very low (Zebe, 1959; Newsholme and Taylor, 1969). Consequently, glycerol concentration in locust flight muscles increased 3-fold within 15 min of flight (Worm and Beenakkers, 1980), and in the haemolymph glycerol concentration increased even 10-fold during a 1 hr flight (Candy *et al.*, 1976). In fact, in flight muscles, haemolymph and fat body steady state levels of glycerol with very high turnover rates are eventually reached (Van der Horst *et al.*, 1983). As mentioned before, in the fat body glycerol is used for re-esterification of fatty acids produced during the flight-induced conversion of TG into DG, while additionally part of the glycerol is used for trehalose synthesis (Candy *et al.*, 1976; Candy, 1981; Van der Horst *et al.*, 1983). Thus, glycerol apparently plays a rather central and integrative role in the metabolism of both lipid and carbohydrate during flight (Fig. 10).

As utilization of fatty acids for energy production cannot proceed under anaerobic conditions, an intimate association exists between fatty acid degradation, the citric acid cycle and the respiratory chain (cf. Beenakkers *et al.*, 1981a). The fatty acid moieties are converted within the mitochondria by the β-oxidative pathway into acetyl-CoA units, which involves a preceding interaction of carnitine to transport the acyl groups across the mitochondrial membrane (cf. Beenakkers *et al.*, 1981*a*, 1984), though the flight muscles of two moth species, *Prodenia eridania* and *Trichoplusia ni*, apparently are exceptions to the requirement of carnitine as an essential factor (Stevenson, 1968). Perhaps, as suggested by Downer (1978), this may be related to a higher body temperature in the latter insects during flight, since permeability of membranes for long-chain fatty acids increases with temperature.

In insects utilizing exclusively carbohydrates during flight, control of flight muscle pyruvate oxidation is exerted at the level of the dehydrogenases, particularly *iso*citrate dehydrogenase (cf. Beenakkers *et al.*, 1984). In species depending on fatty acid oxidation during flight, the question arises as to whether metabolic control is also exerted primarily upon the citrate cycle or directly upon the β-oxidative pathway. In the flight muscle mitochondria of *Manduca sexta*, Hansford and Johnson (1976) implicated *iso*citrate dehydrogenase as a primary control point. In the locust, the regulation of the successive utilization of carbohydrate and lipid is still unclear. Despite the continuous availability of trehalose, fatty acid oxidation increases during flight. Phosphofructokinase, an important regulatory enzyme in the glycolytic sequence in mammalian muscle, is inhibited by high concentrations of citrate resulting from fatty acid oxidation. In locust flight muscles, however, citrate does not inhibit the enzyme (Walker and Bailey, 1969; Newsholme *et al.*, 1977). Up until now, no enzyme in the glycolytic chain has been shown to regulate glycolytic flux in favour of fatty acid oxidation, though recently this role has been suggested for aldolase (Storey, 1980b). Inactivation of pyruvate dehydrogenase by an increase of acetyl-CoA concentration derived from β-oxidation is invalid as in contrast, acetyl-CoA concentration decreased sharply after the onset of flight (Worm *et al.*, 1980). The problem remains to be solved; besides, hormonal stimulation of fatty acid uptake into the flight muscle mitochondria cannot be ruled out (Robinson and Goldsworthy, 1977b; Candy, 1978), but clearly further work is required to understand the mechanism underlying a hormonal control.

6. PROLINE AND INSECT FLIGHT

The role of proline may be different in various insect species, ranging from providing intermediates of the citric acid cycle to acting as a primary energy substrate for flight. The question of why proline participates particularly in flight muscle metabolism has been discussed by Bursell (1981). Considering factors such as yield of metabolic energy, solubility, amount of nitrogen production which requires detoxication at the expense of energy, he concluded that of all potential amino acids proline is the most suited. Besides, mitochondria are able to oxidize exogenous proline, in contrast to glutamate or aspartate (or a number of intermediates of the citric acid cycle) which do not function as a substrate (cf. Sacktor, 1975).

6.1 *General aspects of proline metabolism*

Transition from rest to flight results in a decrease of proline concentration in the flight muscles of many insects, generally with a concomitant rise in alanine concentration, as shown for two species in Fig. 11. Isolated flight muscle mitochondria are capable of

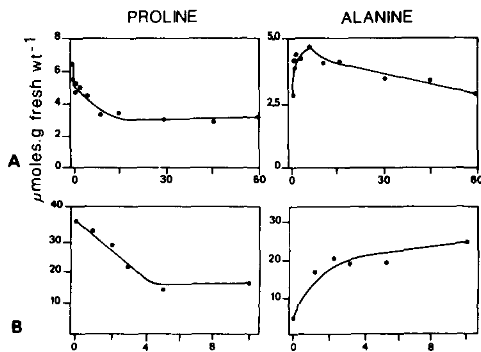

Fig. 11. The concentrations of proline and alanine in the flight muscles of the blowfly (A) and the Colorado beetle (B) (cf. Beenakkers *et al.*, 1984).

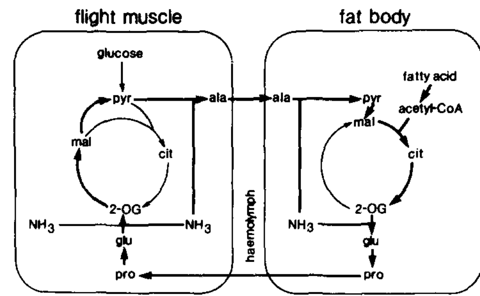

Fig. 12. Schematic representation of the pathways of proline oxidation in flight muscle, and proline synthesis in fat body of tsetse fly and Colorado beetle (adapted from Bursell, 1977, and Weeda *et al.*, 1980b).

proline oxidation, as was demonstrated for the blowfly (Q_{O_2} of 0.2 μg atom O_2 mg protein^{-1} hr^{-1}, Sacktor and Childress, 1967), the Japanese beetle (Q_{O_2} 0.57, Hansford and Johnson, 1975), the tsetse fly (Q_{O_2} 2.69, Bursell, 1975), and the Colorado beetle (Q_{O_2} 1.19, Weeda *et al.*, 1980*a*) (Table 3).

The oxidation of proline to glutamate proceeds in a two-step reaction, the first being catalysed by proline dehydrogenase. Glutamate is then converted to oxoglutarate by either alanine aminotransferase or glutamate dehydrogenase. If the transferase is the exclusive enzyme, stoichiometry exists between proline utilization and alanine production, as is true for the insects shown in Fig. 11, but this does not apply to the migratory locust (Worm and Beenakkers, 1980). Regulation of proline oxidation may occur at the level of proline dehydrogenase, ADP being an allosteric effector, lowering the apparent K_m for proline, as demonstrated in the blowfly (Hansford and Sacktor, 1970); pyruvate also increases the affinity of the dehydrogenase for its substrate. In the tsetse fly the enzyme is inhibited by glutamate (Norden and Venturas, 1972). Glutamate dehydrogenase is potentiated by ADP, but ammonia acts as an inhibitor (Bursell, 1975).

6.2 *Proline as energy substrate*

Proline can participate in flight muscle metabolism as a direct and in some insects most prominent substrate. Outstanding examples are the tsetse fly, *Glossina morsitans*, and the Colorado beetle, *Leptinotarsa decemlineata*. As shown in Table 3, *Leptinotarsa* flight muscle mitochondria oxidize proline at a much higher rate than pyruvate, and in *Glossina* flight muscles this effect is even more pronounced (Bursell and Slack, 1976) (Table 7). Tsetse fly mitochondria converted about 80% of administered radiolabelled proline into alanine, suggesting that about 20% of the substrate is subjected to glutamate dehydrogenase activity, liberating NH_3. In the Colorado beetle a strict stoichiometry exists between proline utilized and alanine produced (Weeda *et al.*, 1980a).

The pathway for proline metabolism in flight muscle is given in Fig. 12. An important step is the synthesis of pyruvate from citric acid cycle intermediates. Hoek *et al.* (1976) demonstrated a very active NAD-linked malic enzyme in various species of *Glossina*. This enzyme is also present in flight muscle preparations of other insects (Table 7). In *Leptinotarsa* flight muscles Weeda *et al.* (1980a) compared the activities of the four enzymes possibly responsible for the pyruvate synthesis and revealed that only the activity of NAD-dependent malic enzyme was sufficiently high to meet the rate of alanine accumulation *in vitro* with isolated mitochondria.

In the proposed pathway of proline utilization only one segment of the citric acid cycle is involved, namely from oxoglutarate to malate. It is therefore of interest to compare flight muscle enzyme activities of the citric acid cycle between insects utilizing proline and insects utilizing another substrate. Norden and Matanganyidze (1979) made such a comparison between tsetse fly and fleshfly; the ratio of each of the enzymes 2-oxoglutarate dehydrogenase, succinate dehydrogenase, and fumarate hydratase between tsetse fly and fleshfly is about 1, whereas for instance that ratio of citrate synthase, *iso*citrate dehydrogenase, and malate dehydrogenase reaches values of about 0.1, clearly indicating that the "second segment" of the cycle is equally important in both flies, but that the "first segment" has much less actual value in the proline utilizing tsetse fly. The diverging activity ratio citrate synthase (CS)/succinate dehydrogenase (SDH) in the Colorado beetle compared to the other insects, noted in Table 2, indicates the same phenomenon in the beetle.

Isolated mitochondria of *Leptinotarsa* flight muscles demonstrate high respiratory rates with proline, but pyruvate is oxidized at appreciable rates as well,

Table 7. Respiratory rate and enzyme activities related to proline oxidation in insect flight muscles

Species	Q_{O_2} proline / Q_{O_2} pyruvate	Proline dehydrogenase	NAD-linked malic enzyme	Alanine aminotransferase
Tsetse fly	76.0	140	340	1340
Blowfly	0.2	23	30	353
Locust	0.1	17	35	334
Colorado beetle*	4.0	—	252	—

Enzyme activities in nmol min^{-1} mg protein^{-1}. From Pearson *et al.* (1979) except* from Weeda *et al.* (1980a).

raising the question of the extent to which both substrates participate in energy generation *in vivo*, Weeda *et al.* (1980a), using mitochondrial preparations, demonstrated that the rates of oxidation of proline and pyruvate are additive only when proline concentration remains below 1 mM. At higher concentrations of the amino acid, pyruvate oxidation is completely turned off (Table 3).

As indicated in Fig. 12 also, resynthesis of proline takes place in the fat body. Both in the tsetse fly (Bursell, 1977), and in the Colorado beetle (Weeda *et al.*, 1980b) this synthetic capacity was shown, revealing that particularly, if not exclusively, alanine and fatty acids take part in proline synthesis. Acetyl-CoA, derived from fatty acids, combines with alanine in the overall equation: alanine + acetyl-CoA + NADPH + $H^+ \rightarrow$ proline + $2\,H_2O$ + $NADP^+$ + CoA. From the more detailed pathway, shown in Fig. 12, it is clear that transamination of alanine provides pyruvate that is then carboxylated to malate, at least in the Colorado beetle (Weeda *et al.*, 1980b). In the tsetse fly Bursell (1977) proposed oxaloacetate as the carboxylation product. Condensation of oxaloacetate with acetyl-CoA, derived from fatty acids, produces citrate that is metabolized to oxoglutarate. After transamination glutamate is reduced to proline. Thus in the fat body, contrary to the flight muscles, the tricarbonic acid section of the citric acid cycle is much more important than the dicarbonic acid section; CS/SDH ratio amounts to 12.3 in Colorado beetle fat body and only to 2.2 in locust fat body (Weeda *et al.*, 1980b). Fatty acid oxidative capacity in beetle fat body exceeds by far that in locust fat body.

In the system described, alanine produced by flight muscle metabolism is transported to the fat body, and reconverted to proline, that may be used again as a flight substrate. Candy (1981) has indicated an analogy between insects utilizing diacylglycerol and those using proline; in the latter insects alanine provides a carrier for fatty acid carbon, whereas for instance in the locust glycerol provides such a carrier. In this respect it is striking that corpora cardiaca extracts enhance proline synthesis in the fat body of both the Colorado beetle (Weeda, 1981), and the tsetse fly (Pimley and Langley, 1982). The proline synthetic rate, calculated in the resting situation of both tsetse fly and Colorado beetle fat body, does not match proline oxidation rate in the flight muscles during flight. If corpora cardiaca hormones are actually released during flight, and raise proline synthetic activity as demonstrated *in vitro*, this discrepancy might be diminished or even abolished. Further investigations on regulation of proline synthesis therefore are of importance.

Isolated flight muscle mitochondria of the Japanese beetle, *Popillia japonica*, can oxidize both proline and pyruvate; respiratory rates with both substrates do not differ substantially (Hansford and Johnson, 1975). The oxidation of proline gave rise to much more NH_3 than alanine, demonstrating the predominance of glutamate dehydrogenase activity. It was shown that the resulting pyruvate was oxidized via the citric acid cycle. Thus in this insect most of the proline is oxidized completely, which is at variance with proline metabolism in the tsetse fly and the Colorado beetle. However, the extent to which proline participates in energy generation in *Popillia* in the normal flight situation is unknown.

6.3 *Proline as a "sparker" for the citric acid cycle*

In several orthopteran, lepidopteran, hymenopteran, and dipteran species proline is considered to provide intermediates of the citric acid cycle, necessary for the enhanced oxidation of acetyl-CoA, derived from a flight-induced increase in degradation of carbohydrate or lipid. Isolated mitochondria from blowfly flight muscles are stimulated to oxidize pyruvate after addition of proline (Sacktor and Childress, 1967). In the blowfly (Sacktor and Wormser-Shavit, 1966), and the locust (Worm and Beenakkers, 1980) an increase in flight muscle pyruvate concentration was measured immediately after the onset of flight, but within a few minutes resting values were reached again, concomitant with a rise in alanine. The role of proline may be summarized as follows: at the initiation of flight the increased flux in glycolysis leads to an increased pyruvate concentration. Proline is oxidized to glutamate that is converted to oxoglutarate while (part of) the pyruvate is converted to alanine. The elevated levels of citric acid cycle intermediates enable an increased oxidation of pyruvate (or other substrates). Hence proline utilization is transient and has an anaplerotic function.

Recently the sparker-function of proline has been questioned by Olembo and Pearson (1982), as they could demonstrate only a small change in the total concentration of citric acid cycle intermediates in the flight muscles of either *Glossina* or the carbohydrate utilizing fleshfly, *Sarcophaga*, during the initial phase of flight. Moreover, the proline-alanine pathway is not in keeping with this role, particularly as the elevated alanine concentration in fleshfly or in blowfly flight muscles is maintained for at least the first minutes of flight (cf. Fig. 11). They assume that the pathway contributes to the energy at take-off in many insects and has been further developed as the major energy source for flight in some species.

7. CONCLUSIONS

Insect flight muscle metabolism involves an integration of biochemical, hormonal and neural mechanisms. The demand for substrates providing energy to the contracting muscles requires activation and control of mobilization and transport of fuel reserves from body stores.

Regarding the utilization of substrates during flight several types of insects can be recognized, including insects exclusively generating energy from carbohydrates, those virtually relying solely on lipid oxidation, a third type utilizing both substrates, and insects in which proline acts as a direct and in some species most prominent substrate. In the present review approaches to identifying species-specific flight substrates and metabolism were briefly discussed on the basis of the relatively few examples examined compared to the vast number of insect species known. Though generalizations are difficult to make, all species studied illuminate both the highly

efficient fuel management and the remarkably high metabolic rate characteristic of insect flight.

Of course, despite the notable progress made, the understanding of insect flight metabolism is still interlarded by important flaws both with respect to the biochemical and regulatory mechanisms underlying flight as well as regarding the comparative aspects. We hope that this review crystallizes current lines of thinking in the research area for those interested in furthering its development.

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