

NUTRITIONAL HOMEOSTASIS IN LOCUSTS: IS THERE A MECHANISM FOR INCREASED ENERGY EXPENDITURE DURING CARBOHYDRATE OVERFEEDING?

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Summary

Maintenance of carbohydrate balance *via* changes in CO₂ output volume was investigated in locusts using a flow-through respirometer. The effect of an imbalance in the dietary protein to digestible carbohydrate ratio on expired CO₂ levels was measured in locusts fed one of two synthetic diets [7% protein, 21% digestible carbohydrate (7:21) and 21% protein, 7% digestible carbohydrate (21:7)]. Additionally, the effect of dietary dilution was investigated by feeding locusts one of two diets with a close-to-optimal ratio of protein to carbohydrate, one containing 7% protein and 7% digestible carbohydrate (7:7) and the other containing 21% protein and 21% digestible carbohydrate (21:21). For insects fed unbalanced diets, a higher CO₂ output volume was measured during feeding on diet 7:21 when compared with insects fed on diet 21:7. Locusts also expired a greater volume of CO₂ during the entire 2 h observation period. This response is consistent with specific metabolic control of carbohydrate balance *via* enhanced respiration. For insects

fed balanced diets, the total volume of CO₂ expired over the duration of a meal was greater for insects fed diet 7:7 than for those fed diet 21:21, although this was due entirely to meals lasting longer on the more dilute diet. However, the basal level of respiration rate was greater for insects fed diet 21:21 and, as a result, over the entire 2 h period, CO₂ output volume did not differ between locusts fed diet 7:7 or 21:21. A possible mechanism for enhanced CO₂ output volume on the nutritionally unbalanced diet was investigated, namely triglyceride/fatty-acid substrate cycling. There was no evidence for the presence of the thermogenic effect of this particular cycle on locusts as a means for dealing with excess ingested carbohydrate.

Key words: nutritional homeostasis, respiration rate, locust, feeding, carbohydrate overfeeding, unbalanced diets, wastage respiration, *Locusta migratoria*.

Introduction

Maintaining nutrient balance is a basic biological function and involves both regulation of feeding behaviour and associated physiological responses. The nutritional requirements of an animal can be viewed as a point in a multidimensional nutrient space (Raubenheimer and Simpson, 1993). When no single nutritionally balanced food is present, or the animal is unable to mix nutritionally complementary foods, then this optimal point is unachievable and the problem arises of ingesting more of some nutrients than required for growth and less of others (Raubenheimer, 1992; Raubenheimer and Simpson, 1994). Diet selection studies have shown that locust nymphs defend an intake ratio of protein to digestible carbohydrate close to 1:1 (Chambers *et al.* 1995).

Recently, the post-ingestive removal of excess ingested protein and carbohydrate in locusts fed nutritionally unbalanced diets was investigated (Zanotto *et al.* 1993). On the

basis of gravimetric studies, there was no evidence of regulation through varying digestive and absorptive efficiencies (Zanotto *et al.* 1993). Instead, excess nutrients were apparently removed after having been absorbed from the gut. In the case of surplus protein, enhanced production of uric acid and other nitrogenous waste products occurred, while for carbohydrate, any excess was partly stored as lipid, but most could not be accounted for in either faeces or body tissues (Zanotto *et al.* 1993). Although not measured directly, such studies suggested that unwanted ingested carbohydrate was respired. Such 'wastage respiration' (Zanotto *et al.* 1993; Raubenheimer and Simpson, 1994) is of particular interest as a mechanism for nutritional homeostasis. It is perhaps analogous to 'facultative diet-induced thermogenesis' (FDIT) seen in mammals (Rothwell and Stock, 1979, 1983), which is known to occur when variations in the level of intake of a

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nutrient result in disproportionate increases in respiration. FDIT is considered to be an adaptation for the regulation of energy balance following overfeeding (Rothwell and Stock, 1979, 1983).

There are various possible means of enhancing respiration which might provide the basis for wastage respiration suggested here. These have been studied mainly in vertebrates and include the presence of substrate cycling (Newsholme and Crabtree, 1976) and the direct effect of hormones on catabolism (see review by Jansky, 1995).

Substrate cycling occurs when two opposing chemical reactions, catalyzed by different enzymes, are simultaneously active (Brooks *et al.* 1982; Newsholme *et al.* 1984; Newsholme and Crabtree, 1976). Each conversion involves the hydrolysis of ATP and the production of heat. Such 'futile' cycles provide no net change in the concentration of either the substrate or the product involved in cycling (Wolfe *et al.* 1987), yet they result in increased net energy expenditure coming from different biochemical routes in order to resynthesize ATP, suggesting that they can perhaps provide a mechanism for respiring excess ingested carbohydrate. Particularly, the triglyceride/free-fatty-acid cycle is potentially important in tissues that are involved in the simultaneous breakdown (lipolysis) and re-synthesis (re-esterification) of triglycerides. In mammals, this cycle links changes in plasma glucose concentration with rates of fatty acid mobilization from the tissues involved in lipid deposition. Brooks *et al.* (1983) showed that during feeding in the mouse there is a twofold increase in the rate of this cycle in adipose tissue and this increase is linked to the removal of excess fuels from the bloodstream. The fat body in insects is analogous to the adipose tissue and liver of vertebrates. It has a central role in lipid metabolism and presents a potential site for the presence of a triglyceride/free-fatty-acid substrate cycle.

Therefore, the aims of the present paper were (i) to verify the occurrence of wastage respiration in locusts by direct measurement of CO₂ output using flow-through respirometry; (ii) to identify when during the feeding cycle such respiration occurs, and (iii) to investigate in detail one possible mechanism for providing enhanced respiration, namely triglyceride/fatty-acid cycling. A more detailed analysis of the relationship between respiration and the microstructure of feeding patterns will be presented separately (S. M. Gouveia, F. P. Zanotto, D. Raubenheimer and S. J. Simpson, in preparation).

Materials and methods

Insects and artificial diets

Experimental insects (*Locusta migratoria* L.) were reared at the Department of Zoology, Oxford University, UK, using seedling wheat and wheat germ as a food source. The experimental insects were collected within 4 h of having ecdysed to the fifth stadium (termed day 0). The mass range of the insects was 400–580 mg for males and 480–660 mg for females, these spanning one standard deviation on either side of the mean mass for the culture.

The four dry, granular, chemically defined diets used were

the same as those employed previously by Zanotto *et al.* (1993). They contained the following proportions of macronutrients: 7% protein, 7% digestible carbohydrate (7:7); 7% protein, 21% digestible carbohydrate (7:21); 21% protein, 7% digestible carbohydrate (21:7) and 21% protein, 21% digestible carbohydrate (21:21). Changes in the level of either of the macronutrients were compensated for by altering the amount of indigestible cellulose added to the diets. The proteins used were casein, peptone and albumin (3:1:1), and sucrose and dextrin (1:1) provided sources of digestible carbohydrate. The diets also contained salts, vitamins, cholesterol and linoleic acid, and are fully detailed by Simpson and Abisgold (1985).

Experimental protocol for measuring carbon dioxide emission

Within 4 h of ecdysis to the fifth stadium, insects of both sexes were collected, weighed and placed in individual clear plastic containers (17 cm×12 cm×6 cm). The containers had an aluminium perch, a water dish and a Petri dish containing one of the four artificial diets (7:7, 7:21, 21:7 or 21:21). The insects were kept at 30 °C under a 12 h:12 h L:D photoregime throughout the experiment. After 2 days, each insect was removed to a similar but smaller box (13.5 cm×7 cm×5 cm), which was the same size as the experimental chambers for respirometry. The diets were replenished, and the insects were kept in these boxes for a further 24 h. On day 3, a single locust at a time was placed in the respirometry chamber and was allowed to settle for 10–30 min before measurement of respiratory rate commenced. The box contained a dish of the same diet as that upon which the insect had been pre-treated for the previous 3 days and a water dish. Baseline measurements for each run were made using the same experimental chambers both at the beginning and at the end of each measurement period. Throughout the 2 h of respiratory measurements (see below), the insects were simultaneously observed for feeding behaviour, locomotion and quiescence. The behavioural observations were entered as markers on the computer-recorded respiratory traces. Respirometry was carried out on four insects per day, each representing one of the diet treatments. The order in which insects were tested was varied systematically to ensure that any effects of time of day were controlled for. Two insects were used sequentially in the morning (between 08:00 h and 13:00 h) and two in the afternoon (between 13:00 h and 18:00 h), the photophase having commenced at 08:00 h and finishing at 20:00 h.

After the 2 h recording session, the insects were removed and weighed to the nearest 0.1 mg. Locusts which did not feed within 2 h were not included in the analyses.

Respirometry

CO₂ production rate was measured continuously using a Sable System TR-3 respirometer (Salt Lake City, Utah, USA). Air temperature was 30±1 °C throughout the observation period. Incoming room air was dried and CO₂ was removed (Drierite/Ascarite/Drierite column) before the air entered the

500 ml experimental chambers. The air leaving the experimental chambers was similarly dried before being directed to the CO₂ analyzer (LI-COR LI-6251). Air flow through the respirometer was regulated by a mass-flow controller (together with a flow-meter) at 150 ml min⁻¹ (Sable Systems), downstream of the experimental chamber and the CO₂ analyzer.

DATA CAN V software (Sable Systems) was used for all data acquisition and analysis of the recordings after data collection in % CO₂ output. Variables derived for statistical analyses (see Fig. 1) were the total body-mass-specific volume (ml g⁻¹) of CO₂ produced during meals (the total area under the trace of CO₂ production in ml g⁻¹ h⁻¹ versus time for periods of feeding; V_{CO_2f}), the total area under the trace excluding feeding periods (V_{CO_2nf}) and the total area under the trace throughout the observation period, including feeding and non-feeding events [$V_{CO_2(f+nf)}$]. Owing to the washout constraints of the respirometer, the CO₂ output during feeding was identified as the area under the curve from the beginning of feeding (based on behavioural observations) until values returned to within 10% of levels recorded when feeding started (baseline levels; see Fig. 1). Because most insects fed more than once, CO₂ output volume was calculated for each meal and then averaged for each insect. All volumes were corrected to STPD.

Experimental protocol for measuring triglyceride/free-fatty-acid cycling

Pilot studies were performed to test how much tritiated water (³H₂O) it would be necessary to inject into the insects, and for how long they should be kept feeding *ad libitum* after the injection, in order that tritium incorporation into the lipid fraction of the fat body could be detected. Owing to technical difficulties involved in detecting tritium incorporation above background levels in insects fed low-carbohydrate diets (diets 7:7 and 21:7), which led to low quantities of fat body, it was only possible to measure cycling in insects fed diets 7:21 and 21:21. Previous gravimetric studies (Zanotto *et al.* 1993) had indicated that wastage respiration was pronounced in locusts fed diet 7:21 but not in insects provided with the nutritionally balanced diet 21:21.

Sixty insects (30 males and 30 females, divided into groups of 12 per day) were given either diet 7:21 or diet 21:21 for 3 days after ecdysing to the fifth stadium (the same period used for the respiratory rate experiments). On day 3, the insects were observed during *ad-libitum* feeding, starting 2 h after lights on. After a 5 min period in which no feeding activities were observed, a sub-group of four insects was injected with 1.85 MBq of tritiated water (³H₂O) in 10 µl of distilled water using a Hamilton syringe (representing a 4% increase in blood volume). Each sub-group was injected 40 min apart, to allow time for later dissection.

After the injection, the insects were again allowed access to food and the latency to the next meal was recorded. If locusts did not begin to feed within 100 min after the injection, they were discarded from the experiment. This period was based on

a mean intermeal interval of approximately 80 min for diet 21:21 with an additional 20 min to allow the insects to settle after injection (Zanotto, 1995). After the insects had been allowed access to food for 5 h following injection, they were weighed and a blood sample was collected from each for calculation of haemolymph specific activity. Next, the insects were dissected and the thoracic fat body and flight muscles were collected. The tissues of three insects (two females with one male, or two males with one female) were pooled. The wet mass of each sample was recorded to the nearest 0.01 mg in a pre-weighed Eppendorf tube and the sample was then frozen to -40 °C and stored for subsequent lipid extraction. The dry mass of the food eaten over the 3 days from ecdysis to dissection was measured for each insect by preweighing and later reweighing the diet dish.

Chemical analysis

The method used for the extraction of free fatty acid (FFA) and glycerol from the fat body and flight muscle was based on that of Brooks *et al.* (1982) and Dobbin (1987). Each tissue sample was homogenized in saline solution (PBS) using a glass hand-held homogenizer. A mixture of 3 ml of chloroform/methanol (2:1) was then added and the sample was vortexed and placed in a shaker (80 revs min⁻¹) for 30 min. Samples were centrifuged at 500 g for 5 min, and the chloroform layer was separated from the aqueous layer. The procedure was repeated for complete extraction of lipid from the tissues. Subsequently, the chloroform layers were washed three times using salt solution (0.05 mol l⁻¹ NaCl plus 0.05 mol l⁻¹ H₂SO₄) to remove all traces of tritiated water not present in the lipid fraction of the tissues. Between washings, the samples were mixed well and centrifuged at 500 g for 5 min. The tubes were subsequently oven-dried at 50 °C. The triglyceride present in the samples was hydrolyzed by addition of 3:1 (v/v) ethanol:KOH (60% w/v) and heated at 70 °C for 3 h in sealed tubes. The samples were cooled and neutralized with 0.5 ml of 6 mol l⁻¹ H₂SO₄. The precipitate was washed using 1.5 ml of petroleum ether, mixed and centrifuged as before. The aqueous layer (containing glycerol) was separated from the organic layer (containing FFAs) and each solution was washed again, mixed and centrifuged for thorough separation. The organic layer was oven-dried (50 °C), a few drops of ethanol were added for solubilization and the sample was measured for radioactive incorporation after an Opti Phase HiSafe scintillation cocktail had been added. The aqueous layer was standardized to a known volume, a sample was taken and the radioactivity in the glycerol fraction was measured after addition of scintillation cocktail.

The rates of fatty acid and glycerol synthesis were calculated from measurements of tritiated water incorporation for each tissue assuming that each glycerol molecule incorporated into triglyceride contains 3.3 hydrogen atoms and each fatty acid molecule contains 13.3 hydrogen atoms of tritium (Dobbin, 1987).

The rate of incorporation of FFA into triglyceride will be three times the rate of incorporation of glycerol phosphate

because three FFA molecules are esterified with one glycerol phosphate molecule. The rate of cycling was calculated according to the following equation (Brooks *et al.* 1983):

$$R_c = 3R_g - R_{fa},$$

where R_c is cycling rate, R_g is the rate of glycerol synthesis and R_{fa} is the rate of fatty acid synthesis.

Cycling rates were determined for both flight muscles and fat body, and rates are expressed as $\mu\text{mol h}^{-1} \text{g}^{-1}$ tissue wet mass.

Haemolymph specific activity was measured using a pooled sample of 3 μl of haemolymph from the same three insects that provided pooled fat body and flight muscle samples. The incorporation of tritium was measured in the scintillation counter after addition of Opti Phase HiSafe scintillation cocktail.

Statistical analyses

All variables were tested for normality (Kolmogorov–Smirnov test) and for homogeneity of variances (Levene's test) before analysis. Data were log-transformed where necessary to meet the assumptions for analysis of variance (ANOVA).

For CO_2 output volume, analysis of covariance (ANCOVA) was performed with feeding time or non-feeding time (for $V_{\text{CO}_2(\text{f}+\text{nf})}$ only) as a covariate and diet and time of day as factors. Feeding time here corresponds to meal duration and thus includes the total number of minutes spent eating within a meal together with intra-meal pauses.

For triglyceride/fatty-acid cycling data, the total amounts of diet eaten, locust wet mass growth (changes in wet mass over 3 days) and latency to the next meal were compared using a two-way ANOVA with diet and sex as main effects. A Student's *t*-test was used to check for differences between control tissue (flight muscle) and test tissue (fat body) for each diet in rates of substrate cycling, free fatty acid and triglyceride/glycerol synthesis. Differences between diets in fat body substrate cycling and synthesis rates were also tested using a Student's *t*-test. An ANCOVA was performed to test the effects of diet on substrate cycling for both flight muscle and fat body using the corresponding tissue's wet mass as a covariate. Additionally, ANCOVA was used to test for differences in wet mass growth for the same insects as used for substrate cycling, and on free fatty acid and triglyceride/glycerol synthesis ($\mu\text{mol h}^{-1}$), using fat body wet mass as a covariate and diet as a factor.

Results

Respiratory rate

Two null hypotheses were tested: (i) that the direction of dietary imbalance had no effect on mass-specific CO_2 output volume (comparison of diets 7:21 and 21:7) and (ii) that dilution of a nutritionally balanced food carried no respiratory costs (comparison of diets 7:7 and 21:21).

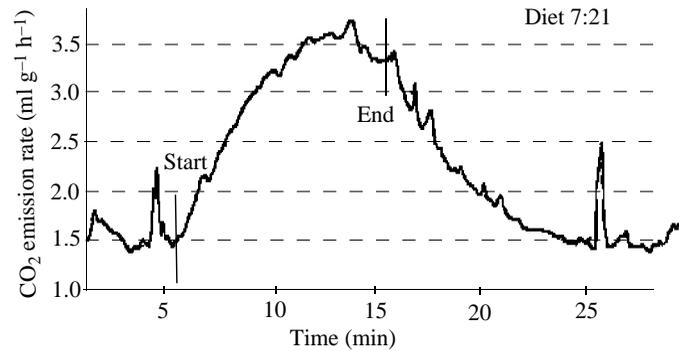


Fig. 1. CO_2 emission rate during a period of feeding for a locust fed diet 7:21 (% protein:% carbohydrate) on day 3 of the fifth stadium. The start and end of feeding behaviour are shown.

Unbalanced diets (7:21 and 21:7)

The elevated rate of CO_2 production during feeding is shown in Fig. 1 in a representative trace for an individual insect fed on diet 7:21. In this case, the meal duration was approximately 10 min (mean \pm S.E.M. for all insects is 12.35 ± 0.911 min, $N=10$). The rapid rise in CO_2 production accompanying the onset of feeding was evident in the representative trace. Note also the rate of return to baseline levels following the end of a meal.

Total body-mass-specific CO_2 output volume [$V_{\text{CO}_2(\text{f}+\text{nf})}$] during the 2 h observation period is shown in Table 1. There were main effects of diet and time on $V_{\text{CO}_2(\text{f}+\text{nf})}$ (Table 2). Insects fed diet 7:21 produced overall 1.7 times more CO_2 than did insects fed diet 21:7 (Table 1). Additionally, the data showed that insects fed both diets had a higher $V_{\text{CO}_2(\text{f}+\text{nf})}$ between 11:00 h and 13:00 h (between 3 and 5 h after the lights were turned on) than during the other periods (Table 2).

Feeding time was significant as a covariate on body-mass-specific CO_2 output volume during feeding ($V_{\text{CO}_2\text{f}}$; Table 2). Insects fed diet 7:21 expired approximately twofold more CO_2 during feeding than did locusts fed diet 21:7 (mean \pm S.E.M. for diet 7:21 = $0.60 \pm 0.08 \text{ ml g}^{-1}$ and for diet 21:7 = $0.29 \pm 0.09 \text{ ml g}^{-1}$) as was shown by a significant diet effect after the covariate had

Table 1. Total body-mass-specific CO_2 output volume for feeding and non-feeding periods [$V_{\text{CO}_2(\text{f}+\text{nf})}$] over the 2 h observation period for locusts fed on different diets

Diet (% protein: % carbohydrate)	<i>N</i>	$V_{\text{CO}_2(\text{f}+\text{nf})}$ (ml g^{-1})
7:21	8	3.74 ± 0.23
21:7	9	2.14 ± 0.11
7:7	9	3.25 ± 0.21
21:21	9	2.99 ± 0.17

Data were collected on day 3 after ecdysis to the fifth stadium. Results of statistical tests are reported in Tables 2 and 3.

N, number of locusts.

Values are means \pm S.E.M.

Table 2. Summary of F-ratios from a two-factor ANCOVA using feeding time or non-feeding time (the latter only for V_{CO_2nf}) as a covariate and diet and time as factors

Source of variation	d.f.	F-value		
		$V_{CO_2(f+nf)}$	V_{CO_2f}	V_{CO_2nf}
Covariate				
Feeding time, f	1	0.16	128.48***	
Non-feeding time, nf	1			49.70***
Main effects				
Diet, D	1	29.88***	8.41*	0.38
Time, T	3	4.83*	4.32*	3.21
Two-way interactions				
D×T	3	0.99	0.17	0.08
D×f	1	2.27	0.88	
D×nf	1			0.93
Residual	8–11			
Total	16–19			

*** $P < 0.001$; * $P < 0.05$.

Note that the residual and total vary according to the variable measured.

The diets compared were 7% protein:21% carbohydrate and 21% protein:7% carbohydrate.

$V_{CO_2(f+nf)}$, body-mass-specific CO₂ output volume during feeding and non-feeding periods (over 2 h measurements); V_{CO_2f} , body-mass-specific CO₂ output volume during feeding period; V_{CO_2nf} , body-mass-specific CO₂ output volume during non-feeding period.

Table 3. Summary of F-ratios from a two-factor ANCOVA using feeding time or non-feeding time (the latter only for V_{CO_2nf}) as a covariate and diet and time as factors

Source of variation	d.f.	F-value		
		$V_{CO_2(f+nf)}$	V_{CO_2f}	V_{CO_2nf}
Covariate				
Feeding time, f	1	1.14	157.26***	
Non-feeding time, nf	1			309.74***
Main effects				
Diet, D	1	1.18	0.10	0.10
Time, T	3	1.06	0.30	0.40
Two-way interactions				
D×T	3	0.39	2.04	2.19
D×f	1	1.05	1.25	
D×nf	1			15.08**
Residual	9–11			
Total	17–19			

*** $P < 0.001$; ** $P < 0.01$.

Note that residual and total vary according to the variable measured.

The diets compared were 7% protein:7% carbohydrate and 21% protein:21% carbohydrate.

See Table 2 for a description of variables.

been taken into account (Table 2). Additionally, the insects respired more between 11:00 h and 13:00 h compared with other periods, as shown by a significant main effect of time of day

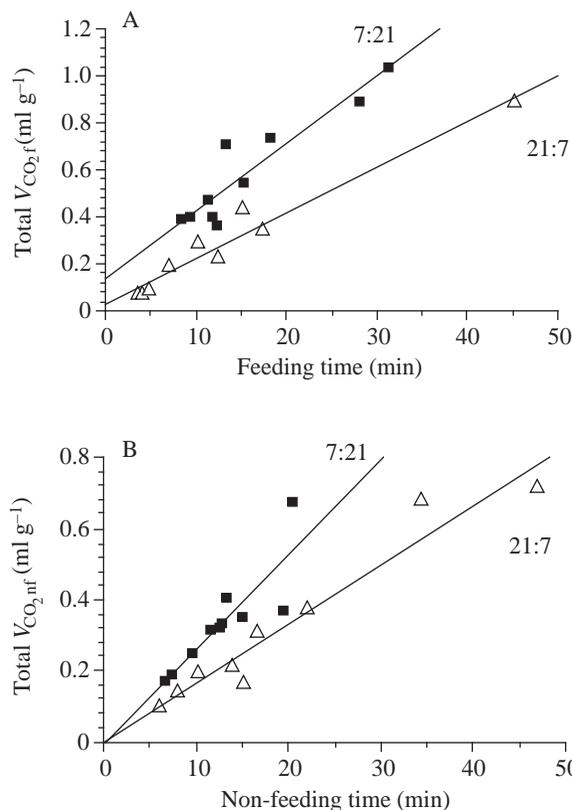


Fig. 2. (A) Mean body-mass-specific CO₂ output volume during feeding (V_{CO_2f}) versus mean feeding time (meal duration) for locusts fed one of two diets (% protein:% carbohydrate) on day 3 of the fifth stadium. The regression equation for diet 7:21 is $y = 0.141 + 0.029x$, $r^2 = 0.87$, $P < 0.001$ and that for diet 21:7 is $y = 0.027 + 0.019x$, $r^2 = 0.96$, $P < 0.0001$. (B) Mean body-mass-specific CO₂ output volume during non-feeding (V_{CO_2nf}) versus mean non-feeding time for locusts fed one of two diets on day 3 of the fifth stadium. The regression equation for diet 7:21 is $y = -0.006 + 0.027x$, $r^2 = 0.76$, $P < 0.01$ and that for diet 21:7 is $y = -0.003 + 0.017x$, $r^2 = 0.94$, $P < 0.0001$.

(Table 2). A plot of V_{CO_2f} versus feeding time shows that the overall CO₂ output during feeding was higher for insects fed diet 7:21, irrespective of the time spent feeding (Fig. 2A; see Table 2 for a significant diet effect).

There was no difference in body-mass-specific CO₂ output volume during non-feeding periods (V_{CO_2nf}) between insects fed diet 7:21 and those given diet 21:7 (Table 2). However, mean V_{CO_2nf} was affected by time spent non-feeding (Table 2). The plot for these two variables against each other illustrates a similar effect for both diets (Fig. 2B; there was no diet effect after the covariate had been taken into account).

Balanced diets (7:7 and 21:21)

There was no significant effect of diet on $V_{CO_2(f+nf)}$ between insects fed diets 7:7 and 21:21 (Tables 1, 3). Additionally, the amount of time spent feeding was not significant as a covariate on $V_{CO_2(f+nf)}$ during the 2 h observation period (Table 3).

V_{CO_2f} was greater for diet 7:7 (mean \pm S.E.M. for diet 7:7 = 0.69 ± 0.12 ml g⁻¹ and for diet 21:21 = 0.31 ± 0.06 ml g⁻¹),

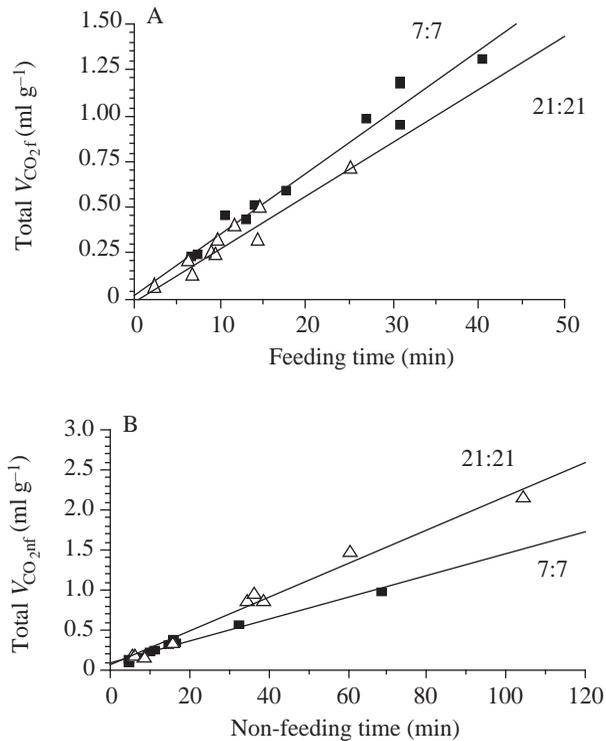


Fig. 3. (A) Mean body-mass-specific CO₂ output volume during feeding (V_{CO_2f}) versus mean feeding time (meal duration) for locusts fed one of two diets (% protein:% carbohydrate) on day 3 of the fifth stadium. The regression equation for diet 7:7 is $y=0.026+0.033x$, $r^2=0.97$, $P<0.0001$ and that for diet 21:21 is $y=-0.009+0.029x$, $r^2=0.91$, $P<0.0001$. (B) Mean body-mass-specific CO₂ output volume during non-feeding (V_{CO_2nf}) versus mean non-feeding time for locusts fed one of two diets on day 3 of the fifth stadium. The regression equation for diet 7:7 is $y=0.094+0.013x$, $r^2=0.97$, $P<0.001$ and that for diet 21:21 is $y=0.069+0.021x$, $r^2=0.98$, $P<0.0001$.

but this effect was due to the longer feeding time on this diet, as indicated by the fact that there was no significant effect of diet when feeding time was included as a covariate (Table 3). Moreover, there was no significant interaction between diet and feeding time (similar slopes; Table 3). A plot of feeding time against V_{CO_2f} is shown in Fig. 3A.

V_{CO_2nf} was not affected by diet (Table 3). However, time spent non-feeding was significant as a covariate on V_{CO_2nf} , and there was a significant interaction between diet and time spent non-feeding (Table 3). A plot of V_{CO_2nf} versus non-feeding time illustrates this interactive effect, showing that insects fed diet 21:21 produced more CO₂ per non-feeding time than did insects fed diet 7:7 (Fig. 3B).

Table 4. Effect of two diets on the time to the next meal after injection of tritiated water, on the mass of diet eaten and on the change in wet mass over 3 days (wet mass growth) after ecdysis to the fifth stadium

Diet (% protein: % carbohydrate)	Latency to next meal (min)	Mass of diet eaten (mg)		Wet mass growth (mg)	
		Male	Female	Male	Female
7:21	46.63±4.05	700.9±37.0***	910.0±31.9***	281.5±18.4	390.3±19.7
21:21	62.70±4.53*	487.6±23.5	595.1±23.9	329.7±17.1*	422.5±20.8*

Values are means ± S.E.M. (N=60).

Asterisks show significant differences between diet mean values based on two-factor ANOVA with diet and sex as main effects; *** $P<0.001$; * $P<0.05$.

Table 5. Rates of free fatty acid and glycerol synthesis and triglyceride/free-fatty-acid cycling (TG/FFA) for fat body and flight muscle of locusts on day 3 after ecdysis to the fifth stadium

Diet (% protein: % carbohydrate)	N	Rate of fatty acid synthesis ($\mu\text{mol g}^{-1} \text{h}^{-1}$)		Rate of triglyceride/ glycerol synthesis ($\mu\text{mol g}^{-1} \text{h}^{-1}$)		TG/FFA cycling rate ($\mu\text{mol g}^{-1} \text{h}^{-1}$)	
		Fat body	Flight muscle	Fat body	Flight muscle	Fat body	Flight muscle
7:21	10	19.62±1.78	1.47±0.13***	1.98±0.26	0.71±0.04***	-13.68±1.40	0.67±0.06***
21:21	10	18.03±1.12	1.08±0.10***	2.07±0.11	0.72±0.05***	-11.81±0.95	1.09±0.12***

Values are means ± S.E.M.

Rates are calculated using tissue wet mass.

There was no significant difference between diets for any of the fat body tissue rates (t , test, $P>0.05$); however, rates for flight muscles compared with fat body were significantly different within each food (t -test, *** $P<0.001$).

N, sample size.

Triglyceride/free-fatty-acid cycling

Data for wet mass growth (change in wet mass over 3 days after ecdysis), amounts of diet eaten and latency to the next meal following injection of $^3\text{H}_2\text{O}$ are presented in Table 4. Insects fed diet 21:21 started to feed later than those fed diet 7:21 (Table 4; ANOVA, $P < 0.05$). There was no sex difference for the initiation of the next meal. The total mass of diet eaten over 3 days during the fifth stadium for males and females is shown in Table 4. Females ate 23% more than males on diet 7:21 and 18% more on diet 21:21 (Table 4; ANOVA, $P < 0.001$). Wet mass for both sexes combined increased by 330 mg for insects fed diet 7:21 and 380 mg for insects fed diet 21:21, and female wet mass increased by 28% more than male wet mass on diet 7:21 and 22% more on diet 21:21 (Table 4; ANOVA, $P < 0.05$).

Rates of triglyceride/free-fatty-acid (TG/FFA) cycling for fat body (test tissue) and flight muscle (control tissue) are shown in Table 5. For the fat body, there was no statistical difference in the rates of fatty acid and triglyceride/glycerol synthesis between insects fed diets 7:21 and 21:21 (t -test, $P > 0.05$). The TG/FFA cycling rate was negative (see Discussion) and not significantly different between diets (t -test, $P > 0.05$). However, rates of TG/FFA cycling, fatty acid and triglyceride/glycerol synthesis differed significantly when fat body and flight muscles were compared (t -test, $P < 0.001$; Table 5). Rates of fatty acid synthesis were approximately 95% lower in flight muscles compared with fat body, whereas rates of triglyceride/glycerol synthesis were approximately 65% lower (Table 5).

The relationship between rates of TG/FFA cycling ($\mu\text{mol h}^{-1}$) and fat body wet mass (mg) is shown in Fig. 4A. There was a strong effect of fat body mass as a covariate on fat body cycling rates (Table 6). Moreover, the rates were proportionately higher for free fatty acid and triglyceride/glycerol synthesis due to the higher fat body wet mass on diet 7:21 compared with diet 21:21 (Fig. 5A,B). The insects had on average 35% more fat body on diet 7:21 compared with diet 21:21 (mean \pm S.E.M. for fat body wet mass on diet 7:21 = 51.43 ± 3.58 mg and on diet

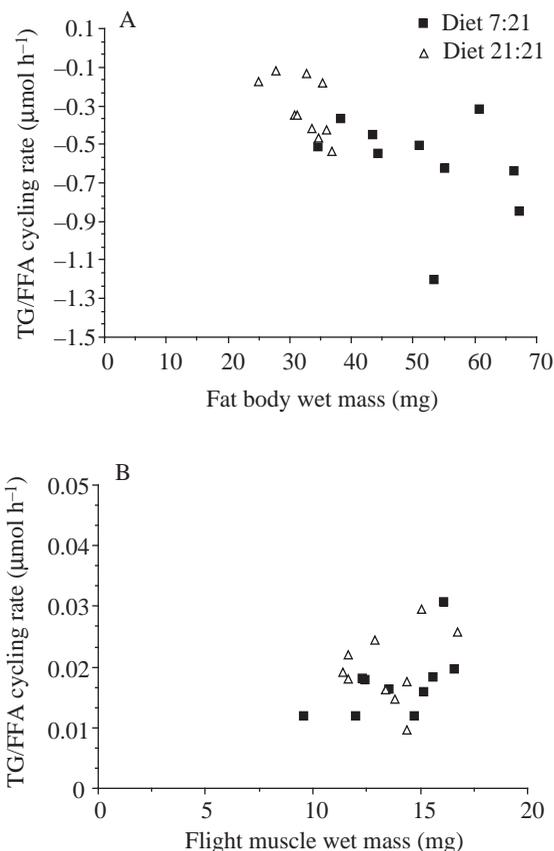


Fig. 4. (A) Relationship between rates of triglyceride/fatty-acid (TG/FFA) cycling and fat body wet mass. (B) Relationship between rates of triglyceride/fatty-acid cycling and flight muscle wet mass. Both rates are measured for diets 7:21 and 21:21 (% protein:% carbohydrate) in *Locusta migratoria* on day 3 of the fifth stadium. $N=10$ for each diet and tissue.

21:21 = 32.51 ± 1.20 mg). The covariate (tissue wet mass) did not have a significant effect when cycling rates for flight muscle were plotted against flight muscle wet mass (Fig. 4B; Table 6).

Table 6. Summary of F-ratios from one-factor ANCOVA using fat body tissue wet mass or flight muscle wet mass as a covariate and diet (7% protein:21% carbohydrate or 21% protein:21% carbohydrate) as a factor for nymphs of *Locusta migratoria* on day 3 of the fifth stadium

Source of variation	d.f.	F-value				
		Fat body cycling rate ($\mu\text{mol h}^{-1}$)	Flight muscle cycling rate ($\mu\text{mol h}^{-1}$)	Free fatty acid synthesis rate ($\mu\text{mol h}^{-1}$)	Triglyceride/glycerol synthesis rate ($\mu\text{mol h}^{-1}$)	Wet mass growth (mg)
Covariate						
Tissue mass	1	12.49**	3.22	31.85***	8.70**	0.61
Main effect						
Diet	1	0.49	1.15	0.00	0.76	33.72***
Residual	17					
Total	19					

*** $P < 0.001$; ** $P < 0.01$.

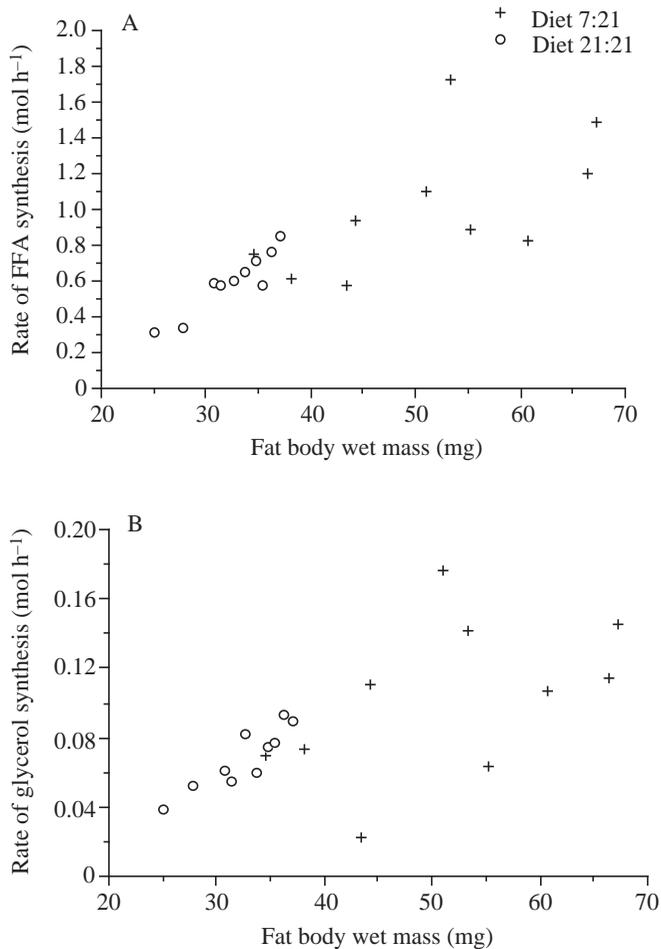


Fig. 5. (A) Relationship between rate of free fatty acid (FFA) synthesis and fat body wet mass. (B) Relationship between rate of glycerol synthesis and fat body wet mass. Both rates are measured for diets 7:21 and 21:21 (% protein:% carbohydrate) in *Locusta migratoria* on day 3 of the fifth stadium. $N=10$.

Discussion

Carbon dioxide emission

Body-mass-specific CO_2 production rate began to increase as soon as feeding commenced, and this increase occurred irrespective of the diet. There was a tight coincidence between CO_2 output increase and the initiation of feeding for all diets. This suggests that the mechanism underlying this response is either directly related to the performance of the behaviour itself or, alternatively, is a rapid response to some sensory stimulus associated with it (see further discussion in S. M. Gouveia, F. P. Zanotto, D. Raubenheimer and S. J. Simpson, in preparation).

Additionally, the physical costs of feeding and its associated processes of salivation, enzyme secretion, digestion and absorption of food from the gut will certainly be associated with enhanced respiration rates. A combination of all these effects could explain the rapid and pronounced change in CO_2 emission rate, although it is interesting to note that neither drinking nor locomotion within the chamber caused changes

in CO_2 production rate as pronounced as those due to feeding (S. M. Gouveia, F. P. Zanotto, D. Raubenheimer and S. J. Simpson, in preparation). Locusts fed on seedling wheat display a similar response during feeding (S. M. Gouveia, unpublished data) to the values reported here, ruling out any mechanical or physiological effects associated with the consumption of artificial diets. It is also unlikely that the rapid rise in CO_2 emission rate reported here is a consequence of the mechanical effects of feeding causing the spiracles to open. Locusts and grasshoppers are known to discharge CO_2 continuously during their primary mode of ventilation (Hamilton, 1964; Hadley and Quinlan, 1993).

An elevation of respiratory rate after feeding is known to occur in vertebrates, but a response as rapid as that found here has not been reported previously. In mammals, a peak in the rate of CO_2 production due to digestion generally occurs 1.5–2 h after feeding. In bats, this lasts for up to 9 h (Morris *et al.* 1994). The same pattern is seen in poikilothermic vertebrates such as fish (Carey *et al.* 1984). In the isopod *Ligia pallasii*, an increase in metabolic rate occurs 2–3 h after feeding (Carefoot, 1990), and even for blood-feeding insects that feed intermittently, such as *Rhodnius prolixus*, a peak in the rate of CO_2 production occurs 3–4 days after feeding (Davey, 1993). In the present experiments, the insects were not starved before measuring CO_2 output, so their natural feeding pattern is illustrated. Locusts take meals very frequently, and the results show that the physiological events associated with their feeding patterns are tightly coupled.

Unbalanced diets

As predicted from previous gravimetric studies (Zanotto *et al.* 1993), an elevated CO_2 output volume was recorded in insects fed diet 7:21. This was evident during feeding but not during non-feeding periods.

The higher CO_2 output volume recorded during feeding on diet 7:21 compared with diet 21:7 is extremely unlikely to be due to differences in the effort involved in eating the two diets. There were no differences in ingestion rate between these diets (Zanotto, 1995) and both contained the same quantity of cellulose.

It is possible that a sensory stimulus associated with feeding and dependent upon the nutritional properties of these two diets elicits processes which consume energy and require differentially enhanced respiration rates. A possible candidate for such a stimulus would be the taste of the food. Sensory stimulation coming from the food is known to increase the rate of CO_2 output in vertebrates (Diamond *et al.* 1985). Palatable foods cause an increased meal-induced thermogenesis in vertebrates compared with non-palatable foods. For locusts, sugars are known to be phagostimulatory (Cook, 1977) up to a certain concentration. Previous work has shown, however, that sugar-saturated locusts, such as those fed diet 7:21, have decreased gustatory responsiveness to sucrose solutions (Simpson *et al.* 1991). Interestingly, it has been suggested that the vole *Microtus ochrogaster* eating an unbalanced low-protein diet increases its metabolic rate to oxidize unwanted

nutrients and in this way is able to enhance the uptake of more limiting nutrients (Trier, 1996).

There are a variety of other possible physiological processes which might be triggered, either neurally or hormonally, by sensory cues associated with the act of feeding that could explain the differential CO₂ output volume between the two diets. These include differential production of digestive enzymes, breakdown and absorption of excess sugar in the gut and the release of neurohormones associated with sugar balance. In locusts, these processes are rapid. Absorption of glucose across the gut is passive and depends on the establishment of a steep concentration gradient through processing of glucose to trehalose by the fat body (Treherne, 1958). While this passive absorption does not seem to be an energetically costly process, the metabolic conversion of glucose to trehalose in the fat body is energy-dependent, with phosphorylation of glucose being the first step in trehalose synthesis (Urich, 1994). It is known that respiration rate in the isolated fat body is linearly related to trehalose synthesis (Steele, 1981). Similarly, fat deposition is an energetically expensive process. Theoretical estimates suggest that the conversion of hexoses to storage fat can take up to 20–25% of the energy content of the food supplied (Westerterp, 1994). Insects fed on diet 7:21 showed an elevated total mass of lipid deposition compared with insects fed diet 21:7 (Zanotto *et al.* 1993), although this process probably occurred after feeding had finished. Additionally, Abisgold and Simpson (1987), using *ad-libitum*-fed locusts provided with similar synthetic diets to those used in the present study, showed that the full meal had emptied from the crop within 30 min of the end of the last meal, which was before the commencement of the next meal. This implies that most of the digestive and absorptive processes involving the excess nutrients eaten were already taking place. Zanotto *et al.* (1996) demonstrated that nutrients from a meal reach the haemolymph very rapidly.

Differences in the respiratory quotient (RQ) between these two diets could potentially account for a large fraction, but not all, of the differences in CO₂ output volume between the diets. The maximal difference in CO₂ output volume which could be accounted for by animals burning pure carbohydrate (diet 7:21) rather than pure protein (diet 21:7) would be 26%, while CO₂ output volume varied by 43% between these diets. Using data for O₂ consumed by each insect during the observation period (data in S. M. Gouveia, F. P. Zanotto, D. Raubenheimer and S. J. Simpson, in preparation), metabolic rates were calculated as $71.128 \pm 5.4392 \text{ J g}^{-1} \text{ h}^{-1}$ for locusts fed diet 7:21 and significantly lower ($P < 0.001$) at $50.208 \pm 3.7656 \text{ J g}^{-1} \text{ h}^{-1}$ for those given diet 21:7. Assuming that carbohydrate and protein are the substrates being respired, these values give mean RQ values of 1.08 and 0.74 for diets 7:21 and 21:7, respectively (S. M. Gouveia, F. P. Zanotto, D. Raubenheimer and S. J. Simpson, in preparation; see Schmidt-Nielsen, 1990). Zanotto *et al.* (1993) determined that insects fed diet 7:21 eat approximately three times more carbohydrate than insects fed diet 21:7, within the same developmental stage used in the present study. Approximating the available energy from the

excess carbohydrate eaten over a 2 h period give values of approximately 468.61 J g^{-1} insect for diet 7:21. The insects respired 18% of this total as CO₂ over 2 h, whereas insects fed diet 21:7 had 17.15 J g^{-1} insect of available energy and respired 5.9% as CO₂. Therefore, a large part of the available energy was probably stored as lipid, glycogen or some other storage compound, although we do not have sufficient information to confirm this.

A higher CO₂ output rate in insects fed high-carbohydrate diets has been reported in blowflies *Phormia regina* (Calabrese and Stoffolano, 1974) and the thermogenic effect of carbohydrate overfeeding is known in vertebrates (e.g. Astrup *et al.* 1986). These higher respiratory rates are, however, usually related to the digestion and assimilation of excess carbohydrate and are not likely to be evident as quickly after the onset of feeding as seen in the present experiments. It is known for vertebrates that a so-called 'facultative' component of diet-induced thermogenesis occurs during overconsumption of food, serving to keep energy balance in check for animals fed a 'cafeteria' diet (Rothwell and Stock, 1979). Such diet-induced thermogenesis can be caused by mechanisms such as increased brown fat tissue activity (Rothwell and Stock, 1979), enhanced rates of substrate cycling (Newsholme and Crabtree, 1976), low-protein diets (see recent work by Trier, 1996) or, alternatively, by insulin-mediated changes in the sympathetic nervous system causing an increase in heat production (see review by Jansky, 1995). Analogously, insects have hypotrehalosaemic factors in the haemolymph with insulin-like properties which regulate haemolymph sugar levels (Kramer, 1985). Interestingly, Zanotto *et al.* (1996) found that insects fed diets high in carbohydrate and low in protein (7:21) have as much as 30–50% higher haemolymph glucose and trehalose levels than insects fed the reciprocally unbalanced diet (21:7). Neuroendocrine hormones have been reported to influence respiration rate in insects (see review by Keeley, 1981).

Balanced diets

There was a difference in mean CO₂ output volume during feeding in insects fed diet 7:7 compared with those fed diet 21:21 due to a longer feeding time on the first diet. The size of a meal is known to alter metabolic responses through increased oxygen consumption in vertebrates (McGregor and Lee, 1995; Carefoot, 1990). Locusts, for example, feed for nearly twice as long during meals on diet 7:7 than on diet 21:21 (Zanotto, 1995). However, taking the feeding time into account, there was no significant difference in the overall volume of CO₂ output during feeding between the two diets.

In addition, CO₂ output volume increased during non-feeding periods in insects fed diet 21:21 as non-feeding time progressed. Perhaps the long-term processes of digestion and assimilation of nutrients (including lipid deposition and storage) in insects fed diet 21:21 contributed to this increase. Previous work has shown that lipid deposition over the fifth stadium is higher for insects fed diet 21:21 than for insects fed diet 7:7 (Zanotto, 1995). Behavioural measurements have shown that locusts fed on diet 21:21 do not show higher levels

of locomotion than insects fed diet 7:7 (Zanotto, 1995), ruling this out as an explanation for increased CO₂ output volumes during non-feeding periods.

Triglyceride/free-fatty-acid cycling

The results suggest that, under the conditions of the present study, locusts do not compensate for excess dietary carbohydrate by utilizing triglyceride/free-fatty-acid cycling.

The presence of substrate cycling has been demonstrated for insects in just two cases. The first to be measured was phosphofructokinase/fructose-diphosphatase cycling in bumblebee (*Bombus affinis*) flight muscle (Newsholme *et al.* 1972). It was proposed that the cycle becomes active when bumblebees are foraging under low ambient temperature. During nectar collection between flowers, when flight is interrupted, the ATP hydrolyzed by substrate cycling generates heat and maintains thoracic temperature sufficiently high for subsequent flight. Further work by Clark *et al.* (1973) found that the rate of substrate cycling in *Bombus affinis* was inversely related to temperature. They did not detect substrate cycling in flight muscles when the ambient temperature was above 24 °C. Additionally, substrate cycling was never present when the bumblebees were flying, only during non-flying periods.

Surholt and Newsholme (1983) identified another substrate cycle in the hawkmoth *Acherontia atropos*. The cycle between glucose and glucose 6-phosphate was measured in flight muscles of moths both at rest and during flight. The cycling rate was increased during flight, and the authors suggested that this provided an increased sensitivity of glucose phosphorylation to changes in the requirements for increased glycolysis in the flight muscles. Although lipid is the primary fuel for flight in this moth, increased rates of glycolysis could be useful when extra power output during flight was required. No significant increase in the cycling rate was found in the fat body in these insects during flight. Substrate cycling has therefore been found in two insect species, apparently fulfilling different metabolic tasks: thermogenesis and increased sensitivity of substrate mobilization.

The intrinsic activity of the fat body as a multifunctional tissue in insects, and as a storage depot, is well known. The present results showed that insects fed diet 7:21 ate more than insects fed diet 21:21, although the change in locust wet mass over 3 days was lower for the former diet. Insects pose interesting questions in relation to the mass-dependent costs of fat storage, as has been discussed for vertebrates (McNamara and Houston, 1990; Witter and Cuthill, 1993). The exoskeleton-imposed pattern of discontinuous size increase in insects might present additional constraints relative to continuously growing animals due to limitations on fat storage space. This suggests that deleterious effects could exist for insects which are fed unbalanced diets and have limited fat storage space.

Methodological criticisms

It is worth mentioning that the nature of the assumptions

made when quantifying fatty acid or triglyceride synthesis has led many workers to conclude that rates of TG/FFA cycling *in vivo* are difficult to determine accurately (Zakim, 1973). The assumptions involved in the measurement of the triglyceride/free-fatty-acid cycle *in vivo* are based on work performed primarily with vertebrates (Brooks *et al.* 1983; Dobbin, 1987). It is assumed that the enzyme glycerol kinase is absent from the tissue under study. In the case of insects, the fat body is known to possess glycerol kinase (Tietz, 1969), unlike adipose tissue in vertebrates (Leboeuf *et al.* 1959). The contribution of this enzyme to rates of cycling should be taken into account because the recycling of glycerol from lipolysis would result in underestimation of the cycling rate. According to parameters provided by Tietz (1969) and assuming that the maximum activity of glycerol kinase in fifth-instar *Locusta migratoria* is similar to that for adults, the activity can be approximated as 5 $\mu\text{mol min}^{-1} \text{g}^{-1}$ wet mass of fat body. This means that the cycling rate would have been underestimated by 33% for diet 7:21 and 25% for diet 21:21. This would adjust cycling rate values to approximately -4.5 and -2.9 $\mu\text{mol h}^{-1} \text{g}^{-1}$ wet mass of fat body for diets 7:21 and 21:21, respectively. These values suggest that the true cycling rate approaches zero.

Another assumption involves a knowledge of the contribution of free fatty acids from sources other than triglyceride in the fat body (Dobbin, 1987; Hansson *et al.* 1987). Any additional fatty acid is likely to come mainly from the haemolymph. However, unlike vertebrates, the main source of circulating lipid in the haemolymph of locusts is diacylglycerol, which is up to 35 times more abundant than free fatty acids (Tietz, 1967). Therefore, if any fatty acid was taken up by the fat body of locusts, this would be negligible in comparison with the total amount of fatty acid synthesized here and overall it would overestimate the amount of fatty acid synthesized, indicating that the true value for the cycling rate presented here is even closer to zero.

Overall conclusions

In summary, flow-through respirometry is a powerful technique enabling direct correlation of behavioural and metabolic events. Insects fed diet 7:7 produced a higher total volume of CO₂ during feeding compared with insects fed diet 21:21, although this was entirely due to a longer feeding time on the former diet. These results contrast with the qualitative response seen for insects fed unbalanced diets, where elevated CO₂ output volumes during feeding on diet 7:21 seemed to reflect specific metabolic control of carbohydrate balance *via* enhanced respiration rates.

Flying insects have the highest known mass-specific metabolic rates and also show high rates of oxygen consumption during pre-flight warm-up (Bartholomew *et al.* 1981). It has been shown recently that leaf-cutting ants reach metabolic rates during leaf-cutting which approach values for insect flight muscle, and the metabolic response due to this behaviour terminates as soon as the performance of the behaviour is completed (Roces and Lighton, 1995). In contrast

to vertebrates, the particular morphological characteristics of the respiratory apparatus of insects probably represent a more efficient mechanism for oxygen uptake, reflecting on the rapidity of the processes associated with this greater aerobic capacity (Suarez *et al.* 1996).

The present study indicates that the 'wastage respiration' suggested previously by Zanotto *et al.* (1993) in locusts does not involve the thermogenic effect of the triglyceride/fatty-acid substrate cycle as a means for dealing with excess ingested carbohydrate. This does not rule out the presence of other substrate cycles which could contribute to an increased metabolic rate. For example, glycogen/glucose-1-phosphate cycling is involved in glycogen deposition and breakdown, and the excess dietary carbohydrate ingested could be used for increased deposition of this storage compound.

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