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FUEL USE IN FLIGHT AND ITS DEPENDENCE ON NECTAR FEEDING IN THE HAWKMOTH *AMPHION FLORIDENSIS*

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Summary

Fuel use varies widely among insects; however, the potential determinants of variation in fuel use have not been explored experimentally. This study examines whether fuel use during tethered flight depends upon feeding status in the nectarivorous hawkmoth *Amphion floridensis*. Fuel use in this study is characterized by the respiratory quotient, measured at intervals during a flight using modified closed-chamber respirometry. Moths were either fed twice daily to satiation with 30 % sucrose or unfed, and their fuel use was measured during flights on the first, third and fifth day after eclosion. Flights lasted up to 30 min, with measurements taken at their onset and at 10 min intervals thereafter. Nectar feeding greatly affected fuel use in *A. floridensis*: fed moths relied primarily on carbohydrate, whereas unfed moths relied almost exclusively on fat reserves. Fuel use did not change during a flight, even when flights lasted 30 min or more. Males were initially more extreme than females in their response to feeding treatment: they burned more carbohydrate when fed and more fat when unfed. By the third day after eclosion, however, fuel use in males and females became identical. Rates of oxygen consumption were uncorrelated with respiratory quotient, were higher in fed moths and declined during a flight. These data indicate that fuel use in this nectarivorous hawkmoth is flexible, that carbohydrate is important as a primary flight fuel and that an understanding of ecological factors, particularly foraging habit, is critical to understanding fuel use in insects.

Key words: hawkmoth, *Amphion floridensis*, flight, fuel use, carbohydrate, fat, respiratory quotient, feeding.

Introduction

Flying insects achieve extremely high metabolic rates using diverse sources of flight energy. A number of classic studies describe the use of different metabolic fuels by different insects, including carbohydrate, fat and protein (for reviews, see Beenakkers et al., 1984; Candy, 1989). A few species emerged clearly as fuel specialists; for example, honeybees (*Apis mellifera*) on carbohydrate (Beenakkers, 1969; Woodring et al., 1993; Rothe and Nachtigall, 1989), tse tse flies (*Glossina morsitans*) on proline (Bursell, 1981) and some Lepidoptera (e.g. *Philosamia cynthia*) on fat (Beenakkers et al., 1975). Other species vary fuel use temporally (e.g. *Locusta migratoria*) or metabolize mixtures of fuel (Van der Horst et al., 1978, 1980; Weeda et al., 1980). The factors that determine fuel use are not well understood. Both phylogeny and ecology are likely to have shaped the metabolic strategies of different species; ecological factors (such as variation in diet quality or foraging opportunity) may also influence fuel use among individuals.

The Lepidoptera appear to employ multiple strategies of fuel use. Whereas a number of studies have indicated that butterflies or moths use exclusively fat during flight (Zebe, 1954; Beenakkers, 1969; Beenakkers et al., 1975; Crabtree and Newsholme, 1972; Bailey, 1975; Saktor, 1975; Ziegler and Schulz, 1986), others have found evidence for carbohydrate oxidizing capability (Stevenson, 1968; Crabtree and Newsholme, 1972; Van Handel and Nayar, 1972; Hansford and Johnson, 1976). Variation among species in adult feeding may contribute to this apparent variation in metabolic capability. Although many species feed on nectar as adults, others emerge without functional mouthparts and rely solely on stored larval fat reserves (Fleming, 1968; Janzen, 1984; Miller, 1996). Variation in fuel use may exist among individuals as well; moths and butterflies that feed on nectar as adults can rely either on stored fat or on incoming nectar sugars during flight. Because the net ATP yield from sugars is approximately 20 % higher when they are oxidized directly than when they are converted to fat prior to oxidation (Suarez et al., 1990), one might predict that nectar sugars should be burned preferentially when available. In insects, however, the determinants of fuel use at the species and individual level are almost entirely unexplored.

The hawkmoths (Sphingidae) are an excellent family for investigating fuel use. They are typified by active nectivores, but also include species that do not feed as adults (Fleming, 1968;

Miller, 1997). Their metabolic rates in flight are among the highest recorded (Bartholomew and Casey, 1978; Bartholomew, 1981; Casey et al., 1985), suggesting that they may use carbohydrates to support flight as do other 'high-performance' insects (Candy et al., 1997). However, carbohydrate reliance during flight by hawkmoths or any other Lepidoptera has not been demonstrated. Although starved *Manduca sexta* use nearly exclusively fat during flight (Ziegler and Schulz, 1986), the use of carbohydrates by fed *M. sexta* in pre-flight warm-up suggests their potential use in flight as well (Joos, 1987). In addition, muscle mitochondria from *M. sexta* readily oxidize both pyruvate and palmitoyl carnitine (Hansford and Johnson, 1976), indicating the capability to use either carbohydrate or fat as a fuel for flight. Among the Lepidoptera, therefore, nectarivorous hawkmoths are prime candidates for investigating fuel use flexibility and its dependence on feeding status.

Here, I explore flight fuel use in *Amphion floridensis*, a diurnal, nectar-feeding hawkmoth, by measuring the respiratory quotient (RQ). RQ is the ratio of metabolic CO_2 produced to O_2 consumed, usually measured by proxy as the whole-organism respiratory exchange ratio. Because flights were of extended duration and because insect flight muscle is exclusively aerobic, I assume respiration and metabolism to be in steady state (Winter et al., 1998). Under these circumstances, the respiratory exchange ratio and RQ are equivalent (Walsberg and Wolf, 1995). RQ equals 1 when carbohydrates are being oxidized, whereas RQ is approximately 0.7 during fat oxidation (the exact value is dependent upon the fatty acid mixture being metabolized).

In this study, I use variation in RQ to measure the relative importance of carbohydrates and fats for fueling flight metabolism. Intermediate RQs can result either from protein metabolism or from the use of a combination of fuels; here, I assume that intermediate values indicate the concurrent metabolism of fatty acids and carbohydrates. Because protein is exceedingly limited in the diets of most adult Lepidoptera and is critical for reproduction, it is unlikely that larval protein reserves would be broken down simply for flight metabolism. Amino acids were oxidized slowly and with poor respiratory control by mitochondria isolated from the nectar-feeding moth *Prodenia eridania* (Stevenson, 1968), making their use as fuel additionally unlikely. Using RQ during flight as an index of carbohydrate *versus* fat metabolism, therefore, I explore the effects of nectar feeding, sex, age and flight duration on fuel use. This study therefore provides a first step towards an understanding of how nectar foraging may affect flight metabolism in the Lepidoptera.

Materials and methods

Adult *Amphion floridensis* (Clark) were trapped using bait (Platt, 1969) in Princeton, NJ, USA, during the summer of 1995. They were housed in a 0.6 m×0.9 m×1.2 m flight cage, and provided with potted host plant for oviposition and with 30 % sugar solution. Eggs were removed from the host plants daily. Larvae were reared in 14 cm diameter plastic dishes on freshly collected host plant (Family Vitaceae), primarily wild grape (*Vitis novae-angliae*) but also fox grape (*Vitis labrusca*), European ampelopsis (*Ampelopsis brevipedunculata*) and Virginia creeper (*Parthenocissus quinquefolia*). Adults, eggs and larvae were kept at 27 °C with a 16 h:8 h L:D photoperiod. Relative humidity was maintained at 70–80 %.

Pre-pupae were removed from dishes and allowed to burrow into darkened boxes of moist peat moss. *Amphion floridensis* overwinters as pupae; pupae were therefore stored at 4 °C. Experimental adults emerged the following summer 10–14 days after being returned to 27 °C and a 16 h:8 h L:D photoperiod. Males and females were housed separately in 0.6 m×0.6 m×0.6 m cages. *A. floridensis* is diurnal/crepuscular; therefore, moth cages were kept darkened to prevent activity and excessive wing damage. Moths were misted twice daily with water and were observed drinking from the cage mesh.

Experimental design

Moths were chilled and weighed at eclosion and again at the end of the experiment. Equal numbers of male and female moths were assigned to feeding treatments and individually hand-fed twice daily to satiation from Eppendorf tubes. 'Fed' moths were given 30 % (w/w) sucrose solution, and intakes were determined by weighing the Eppendorf tubes before and after feeding. 'Unfed' moths were given water only; this treatment controlled for the amount of handling received and prevented dehydration. Moths were flown on the first day after emergence (N=46); a subset of these moths was also flown on the third and fifth days after emergence (N=18). Feeding, sex and age were all tested for their effect on fuel use. Moths of different mass were assigned evenly across age and feeding treatments, so that each treatment contained moths of a similar range of sizes.

Tethered flight protocol

Freshly eclosed experimental adults were briefly chilled on ice and weighed. A small patch of scales was gently rubbed away on the thorax, and a colored, numbered bee tag (Chr. Graze K.G.) was fixed to the thorax using a flexible adhesive (Ruscoe livestock identification cement). Inflexible adhesives (such as cyanoacrylate) impeded the thorax flexion required for normal flight. Tags were modified with a small 30 gauge copper loop for attaching a tether. Tags and adhesive weighed less than 10 mg.

Moths were always flown 20–30 min after being fed. To begin a flight, a moth was removed from its cage, and a piece of 30 gauge copper wire approximately 10 cm long was hooked through the ring on its identity tag. If the moth was not already warming up by shivering, it was gently stroked until it initiated pre-flight warm-up (Joos, 1987; Bartholomew et al., 1981). Moths were housed and flown at room temperature (22–26 °C) and approximately 40–45 % relative humidity (measured using a Fisher thermo-hygro digital sensor). Temperature, humidity and barometric pressure were recorded before each flight.

Once a moth initiated flight, its wire tether was attached to a swivel on the inside of the lid of a respirometry chamber

(1.715 l), the lid was lowered onto the chamber, and the chamber was sealed. An initial air sample was withdrawn with a 60 ml syringe *via* a three-way valve (Bartholomew and Casey, 1978) after pumping the syringe to mix the chamber air completely. The chamber was rotated to keep the moth flying steadily; vertical stripes provided an optomotor stimulus (Farina et al., 1995), and a thin coat of Fluon (Northern Products) prevented the moths from alighting on the sides of the chamber. Moths flew steadily for approximately 3 min, and a second air sample was then withdrawn. The time elapsed between the air samples was recorded precisely. The difference in gas concentrations between the two samples allows a single measurement (integrated over 3 min) of respiratory quotient. A subset of RQ measurements (approximately 85 %) also allowed calculation of the rate of oxygen consumption. Although moths that paused briefly in the chamber were included in the RQ analysis, rates of oxygen consumption were calculated only for those moths that flew without pause and for which chamber residence was accurately timed. Moths that beat their wings weakly or hung at the end of the tether were removed from the experiment.

Moths were not directly handled once flying, but were removed from the chamber *via* their short wire tether. The tether was then attached to a long thread leash, and moths were kept flying by being 'walked' for a period of 10 min before being returned to the respirometry chamber for a second respirometric measurement. Sequential respirometric measurements were thus taken at 10 min intervals of flight for as long as the moth remained flying. Depending on how long the moth was willing to fly, therefore, between one and four measurements of RQ and rate of oxygen consumption were collected during flights lasting up to 30 min. Flights ended when moths hung at the end of their tether or ceased to beat their wings for more than a brief period.

This modification of closed-chamber respirometry allowed a relatively large chamber to be used without the high flow rates required by a flow-through system. It also resulted in longer flights, presumably because of the variety of stimuli and freedom of movement allowed during the 10 min intervals between measurements. While on a thread leash, moths exhibited directional, self-motivated flight, including hovering, increases in height and abrupt changes of direction. This method sacrificed minute-by-minute recording of fuel use changes to gain a broader overall picture of fuel use during longer flights.

Sample analysis

Samples were loaded into a background stream of water- and CO_2-free air supplied by a pressurized cylinder and regulated to a flow of 15 ml min^{-1} with a high-precision diaphragmatic flow controller (Porter Instruments). The background airstream served both as a vehicle for carrying the sample through the analyzers and as zero and span gas for the CO_2 and O_2 analyzers, respectively. Samples were injected through a 5 ml water scrubber (Magnesium Perchlorate) into a 22.8 m sample loop of 3 mm o.d. copper tubing (loop volume 30 ml), open to vent at the other end. The entire 60 ml sample was injected, which was shown to flush room air completely, leaving the sample loop full of pure sample. The sample loop was then switched into the background airstream using an eight-way valve (Valco Co.). The background and sample loops were of equivalent length and volume; therefore, their exchange caused only a slight, momentary pressure fluctuation. All plumbing upstream of the analyzers was performed using 3 mm o.d. (1 mm i.d.) copper tubing. The use of the eight-way valve improved upon earlier techniques for sample injection by ensuring that each sample introduced into the background airstream was of identical volume, that there was no mixing of background and sample gas, and that sample injection did not generate a pressure surge at the analyzers.

O_2 and CO_2 concentrations were measured using an AMETEK S3-A single-channel O_2 analyzer and a Licor 6251 CO_2 analyzer, respectively. Signal conditioners (Sable Systems) improved the resolution of voltage output from both analyzers, especially that of the S3-A. The O_2 analyzer was spanned using air taken from outside the building and assumed to be 20.94 % O_2. The resolution of the oxygen analyzer operating in offset mode was $\leqslant$0.002 % O_2, measured as the standard deviation of O_2 readings around the mean for a well-mixed sample. The carbon dioxide analyzer (Licor 6251) was spanned with a certified mix of 0.2 % CO_2 in air, and its resolution was better than 1 p.p.m. or 0.0001 %. Data were recorded on a Dell 386 computer using Datacan V data-acquisition hardware and software (Sable Systems), including a 16-bit A/D card to digitize analog analyzer output. Excurrent gas flowed through a flow meter connected to the computer, allowing the flow rate to be continuously recorded. The electronic flow meter was frequently calibrated with a bubble flow meter over flow rates from 5 to 100 ml min^{-1}. Values of flow rate varied across a recording by a standard deviation of less than 0.1 ml min^{-1}. All components downstream of the eight-way valve were purchased from and/or manufactured by Sable Systems (Henderson, NV, USA).

Calculation of respiratory quotient

The low internal volume of the O_2 analyzer prevented mixing of sample and background gases; therefore, sample [O_2] could be read directly from the data trace. The CO_2 analyzer allowed some mixing of sample and background; therefore, [CO_2] traces were integrated with respect to time, and time was then divided out to recover actual [CO_2]. Sample 'time' is the quotient of sample volume/flow rate; because both were known with high accuracy, actual [CO_2] could be calculated. This calculation serves the same function as a *Z* transform (Bartholomew et al., 1981), but was found to recover [CO_2] more reliably. The calculation of [CO_2] in this way introduced considerably less error than the use of integrals from both the CO_2 trace and the more noisy O_2 trace. All data trace analyses were performed in the Datacan V data analysis program (Sable Systems), using options for baselining, smoothing, means and integration. O_2 and CO_2 concentrations were then compared between initial and final air samples to

determine $\Delta[O_2]$ and $\Delta[CO_2]$ for each segment of flight in the respirometry chamber. RQ is calculated simply as $\Delta[CO_2]/\Delta[O_2]$. These methods of calculation were extensively tested for robustness and introduced potential errors of less than ±0.02 into RQ measurements.

Calculation of rate of oxygen consumption

Differences between individuals and across flights in flight expenditure may be important to the interpretation of patterns of fuel use. Rates of oxygen consumption are therefore calculated; however, because they are measured for enclosed and tethered flight, they are probably not equivalent to metabolic expenditure in free-flying animals (Rayner and Thomas, 1991). The rate of oxygen consumption was calculated as the difference in $[O_2]$ between the initial and final air samples divided by the time (t, min) elapsed between samples, multiplied by the chamber volume (V_c, ml) to give the rate of oxygen consumption ($\dot{V}_{O_2}$) in units of $ml\,O_2\,min^{-1}$:

$$\dot{V}_{O_2} = (\Delta[O_2]/t) \times V_c\,. \quad (1)$$

In a similar study, Bartholomew and Casey (1978) adjusted chamber volume (giving $V_{c,adj}$) to account for the volume of water vapor removed prior to sample analysis:

$$V_{c,adj} = [V_c - (V_c \times P_s \times RH)/P_b]\,, \quad (2)$$

where P_s is the saturated water vapor pressure, RH is the relative humidity, and P_b is the barometric pressure in mmHg (1 mmHg = 0.1333 kPa). Performing this vapor correction changed $\dot{V}_{O_2}$ measurements by less than $0.01\,ml\,O_2\,min^{-1}$ or approximately 1 %. Because inter- and intra-individual variations in rates of oxygen consumption were considerably greater than 1 %, the correction had no effect on patterns in the data. Because accurate barometric pressure readings were not available for all flights, water vapor corrections were not used in these calculations.

Statistical analyses

All statistical analyses were performed in JMP version 3.1 (SAS Institute). Figures show least-square means ± S.E.M. In total, 185 measurements of RQ were collected, 157 of which also provided usable oxygen consumption data. The effects of feeding treatment, sex and day flown were tested separately on RQ and $\dot{V}_{O_2}$ using a nested factorial analysis of covariance (ANCOVA). Flight duration at the time of sampling was included as a continuous covariate. The model removed variation among moths by including moth identity as a random effect, nested within sex and treatment. Sex and treatment effects are therefore tested over a synthetic denominator, which includes random inter-moth variation in its calculation of error. The full factorial model including all three-way interactions was tested initially; however, for the final model, non-significant (P>0.30) three-way interaction terms (and those non-significant two-way interaction terms that did not participate in significant three-way interactions) were removed to provide more power for estimating significant effects. Differences among variable states were tested using linear contrasts. Although the exact time at which a moth ceased flying was not noted, the time at which its last usable air sample was collected provides an index of minimum total flight duration. Effects of feeding treatment, age and sex on time of last sample were tested using a full factorial analysis of variance (ANOVA). Decrease in body mass was also analyzed for the effects of sex, age and feeding treatment with a full factorial ANOVA. All other standard statistical tests are noted in the text. Means are presented ± S.E.M. unless otherwise noted. F-ratios from ANOVA are presented as $F_{d.f.,N}$, where d.f. is degrees of freedom and N is sample size.

Results

Fuel use (respiratory quotient)

Measurements of respiratory quotient ranged from 0.70 to 0.97, indicating that fuel use in *Amphion floridensis* varied continuously from exclusively fat use to exclusively carbohydrate (Fig. 1, mean RQ=0.84±0.005, N=185). The most striking effect in the model was that of feeding treatment: fed moths used significantly more carbohydrate than unfed moths (Fig. 1; Table 1; $F_{1,185}$=144.6, P<0.0001). If we consider an RQ of 0.85 to indicate equivalent contributions of fat and carbohydrate oxidation to supporting flight metabolism (Gollnick, 1988), then carbohydrate was the primary flight fuel in approximately 84 % of all RQ measurements in fed moths (Fig. 1).

Although fed moths had higher RQs than unfed moths across all levels of all factors, there was a highly significant three-way interaction between feeding treatment, sex and age on RQ (Table 1; $F_{2,185}$=9.24, P=0.0002). On the first day after eclosion, males showed a more marked response to feeding than did females: fed and unfed males approached the extremes of carbohydrate use and fat use, respectively, whereas RQ values for females were more intermediate (Fig. 2, all linear

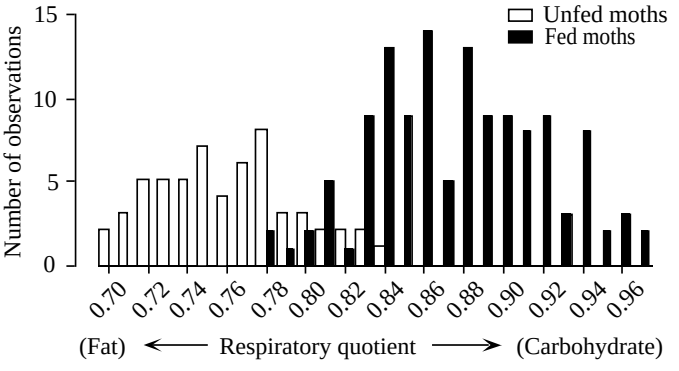

Fig. 1. All observations of respiratory quotient, grouped by feeding treatment (N=185). Filled columns denote measurements from fed moths, whereas open columns denote measurements from unfed moths. Respiratory quotients (RQ) were measured using closed-chamber respirometry, as described in the Materials and methods. RQ=1 indicates exclusive use of carbohydrate, whereas RQ of approximately 0.7 indicates exclusive use of fat. The experimental error in RQ calculation is estimated to be ±0.02.

Table 1. *Results of a nested analysis of covariance examining the effects of feeding treatment, sex, age and flight duration on respiratory quotient*

Effect	SS	d.f.[1]	*F* ratio	*P*
Feeding treatment	**0.1601**	**1**	**144.60**	**<0.0001**
Sex	2.67×10^{-6}	1	0.002	0.9628
Age	4×10^{-5}	2	0.03	0.9753
Flight duration	1.09×10^{-3}	1	1.39	0.2399
Feeding treatment × sex	8×10^{-5}	1	0.07	0.7984
Sex × age	5.8×10^{-4}	2	0.37	0.6916
Feeding treatment × age	**0.01284**	**2**	**8.25**	**0.0004**
Feeding treatment × flight duration	1.5×10^{-4}	1	0.20	0.6580
Age × flight duration	2.21×10^{-3}	2	1.42	0.2457
Sex × feeding treatment × age	**0.0144**	**2**	**9.24**	**0.0002**
Feeding treatment × age × flight duration	$\mathbf{5.88\times10^{-3}}$	**2**	**3.78**	**0.0256**
Moth (feeding treatment, sex)[2]	**0.0830**	**43**	**2.48**	**<0.0001**
Error	7.79×10^{-4}	123		

A factorial model to three degrees was tested initially, and non-significant three-way interaction terms were removed to provide more power for testing significant effects.

[1]*N*=185 samples.

[2]Moth identity was included as a random variable nested within feeding treatment and sex. *F* ratios for feeding treatment and sex were therefore tested over a synthetic denominator as described in Materials and methods

SS, sum of squares; d.f., degrees of freedom.

Significant effects are emphasized in bold type.

contrasts significant at $P<0.002$). By days 3 and 5, however, differences between the sexes in fuel use had disappeared (Fig. 3). RQ declined in all fed moths between day 3 and day 5 (linear contrasts, all $P<0.01$) and stayed constant in unfed moths. This difference is reflected in the significant interaction between feeding and age (Table 1; $F_{2,185}=8.25$, $P=0.0004$).

Flight duration had no systematic effect on RQ (Table 1; $F_{1,185}=1.39$, $P=0.24$). The three-way interaction between flight duration, feeding treatment and age was significant, although not highly significant (Table 1; $F_{2,185}=3.78$, $P=0.0256$), indicating a tendency for RQ to increase during a flight in fed moths on day 1 (slope $0.0012\,\text{min}^{-1}$), a tendency that reverses by day 3 (slope $-0.0019\,\text{min}^{-1}$) and disappears by day 5. Unfed moths showed no change in RQ during flights. Fed moths tended to fly for longer than unfed moths (average last sample at 19 *versus* 7 min of flight, $F_{1,75}=43.04$, $P<0.0001$). The effect of feeding treatment on flight duration interacted significantly with age ($F_{2,75}=3.57$, $P=0.0335$): flight duration increased with age in fed moths, whereas it decreased with age in unfed moths.

Rates of oxygen consumption

The rates of oxygen consumption measured in this study ranged from 12.8 to $56.6\,\text{ml}\,O_2\,\text{h}^{-1}$ (mean $31.1\pm0.436\,\text{ml}\,O_2\,\text{h}^{-1}$, $N=157$). Moths were weighed at eclosion and immediately after their last flight, generating rates of mass loss characteristic of the different treatments (see below). From these rates, the mass of moths in each treatment category at each flight was estimated, yielding a mean mass for all flights of 0.468 g. The estimated body-mass-specific metabolic rate for *Amphion floridensis* was therefore $55.84\,\text{ml}\,O_2\,\text{h}^{-1}\,\text{g}^{0.77}$, using the allometric exponent for hawkmoth metabolic rates to scale mass (Packard and Boardman, 1987; Bartholomew and Casey, 1978). An ANCOVA of last flights only (either day 1 or day 5) revealed no association between rate of oxygen consumption and body mass when the effects of sex, age and feeding treatment were removed (Table 2). Therefore, further analyses involve rates of oxygen consumption that are not mass-specific (the full oxygen consumption rate data set).

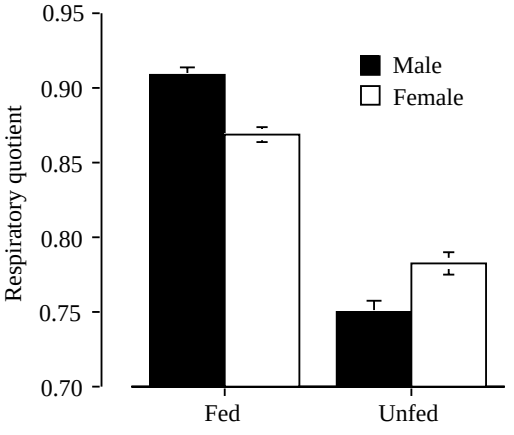

Fig. 2. Response of respiratory quotient (RQ) to feeding treatment in 1-day-old males (filled columns) and females (open columns). Values are least-square means ± S.E.M. from the full RQ ANCOVA model for the term feeding treatment × sex × age; only data from the first day after eclosion are presented. All linear contrasts are highly significant at $P<0.002$.

Table 2. *Results of an analysis of covariance examining the effects of body mass, feeding treatment, sex and age on rate of oxygen consumption*

Effect	SS	d.f.[1]	*F* ratio	*P*
Body mass	3.259	1	0.057	0.8133
Feeding treatment	203.361	1	3.552	0.0707
Sex	190.137	1	3.321	0.0799
Age	46.888	1	0.81	0.3738
Error	1488.43	26		

Interaction terms were non-significant and were omitted from the final analysis.

[1]*N*=31 flights. Data include only the first measurement of rate of oxygen consumption for flights immediately preceding a moth's removal from the experiment, because these are the only flights for which accurate body mass measurements were available.

SS, sum of squares; d.f., degrees of freedom.

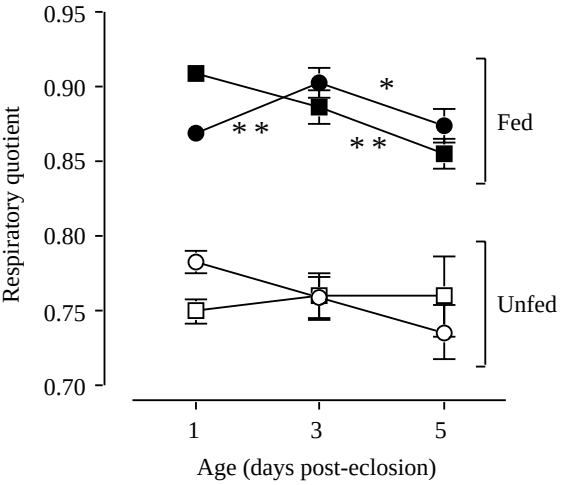

Fig. 3. Effect of increasing age on fuel use in fed (filled symbols) and unfed (open symbols) male (squares) and female (circles) moths. Values are least-square means ± S.E.M. generated by the respiratory quotient ANCOVA model for the term feeding treatment × sex × age. Asterisks denote a statistically significant difference between the means joined by the line adjacent to the asterisk; ***P*<0.007, **P*=0.012 (linear contrasts).

Rates of oxygen consumption were on average 14.6 ml O_2 h^{-1} higher in fed moths than in unfed moths, a highly significant difference (Table 3; $F_{1,157}$=19.57, *P*<0.0001). The difference between the feeding treatments increased with age: rates of oxygen consumption in fed moths increased from day 1 to day 5, whereas rates of oxygen consumption in unfed moths decreased (Table 3; $F_{2,157}$=8.73, *P*=0.0003). The significant three-way interaction between feeding treatment, age and sex indicates that males are primarily responsible for the reduction in rate of oxygen consumption with age in unfed moths (Fig. 4; $F_{2,157}$=3.43, *P*=0.036). Within a flight, the rate of oxygen consumption decreased significantly with increasing flight duration (Table 3; $F_{1,157}$=37.90, *P*<0.0001). This decrease was affected by feeding treatment (Table 3; $F_{1,157}$=9.60, *P*=0.0025): the rate of oxygen consumption in unfed moths dropped more steeply during a flight than that in fed moths. These relationships are presented in Fig. 5, in which adjusted rates of oxygen consumption are plotted against flight duration for fed and unfed moths. Adjusted rates of oxygen consumption were

Table 3. *Results of a nested analysis of covariance examining the effects of feeding treatment, sex, age and flight duration on rate of oxygen consumption*

Effect	SS	d.f.[1]	*F* ratio	*P*
Feeding treatment	**1049.9**	**1**	**19.57**	**<0.0001**
Sex	39.8	1	0.74	0.3918
Age	87.3	2	2.56	0.0824
Flight duration	**646.1**	**1**	**37.90**	**<0.0001**
Feeding treatment × sex	22.3	1	0.42	0.5215
Sex × age	**151.0**	**2**	**4.43**	**0.0144**
Feeding treatment × age	**297.7**	**2**	**8.73**	**0.0003**
Sex × flight duration	0.9	1	0.06	0.8147
Feeding treatment × flight duration	**163.6**	**1**	**9.60**	**0.0025**
Feeding treatment × sex × age	**117.0**	**2**	**3.43**	**0.0363**
Feeding treatment × sex × flight duration	30.4	1	1.78	0.1850
Moth [feeding treatment, sex][2]	**5730.5**	**42**	**8.00**	**<0.0001**
Error	1687.9	99		

A factorial model to three degrees was tested initially, and non-significant three-way interaction terms were removed to provide more power for testing significant effects.

[1]*N*=157 samples.

[2]Moth identity was included as a nested random variable as with the respiratory quotient model (see Table 1) and as described in the Materials and methods.

SS, sum of squares; d.f., degrees of freedom.

Significant effects are emphasized in bold type.

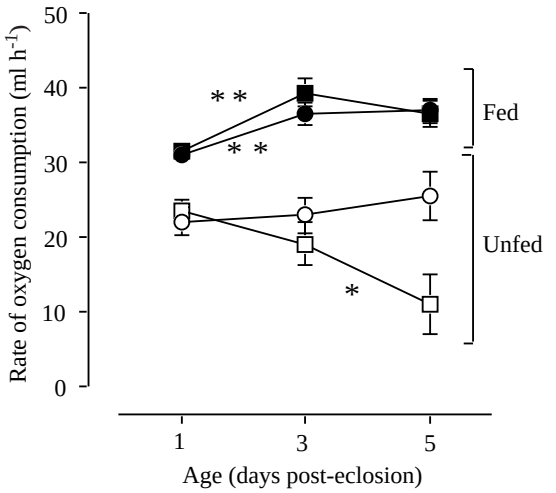

Fig. 4. Effect of increasing age on the rate of oxygen consumption in fed (filled symbols) and unfed (open symbols) male (squares) and female (circles) moths. Values are least-square means ± S.E.M. from the oxygen consumption rate ANCOVA model for the term feeding treatment × sex × age. Asterisks denote a statistically significant difference between the means joined by the line adjacent to the asterisk; ***P*<=0.001, **P*<0.039 (linear contrasts).

generated as the residuals in rate of oxygen consumption after the effects of sex, age and individual had been removed using ANOVA. These residuals were added to the grand mean to give their values biological meaning. These values are analogous to least-square means for categorical variables, but allow all the data to be plotted in a continuous fashion.

The rate of oxygen consumption did not covary with respiratory quotient. Each was tested as a covariate in the analysis of the other; in neither case was there a significant relationship and subsequently each was removed from the other's model.

Body mass

Body mass at eclosion was 0.650±0.024 g in females and 0.500±0.032 g in males. There were no differences in eclosion mass between feeding and age categories. Body mass tended to decline over time, and mass loss was significantly affected by feeding, sex, age and mass at eclosion (Table 4). Declining body mass is characteristic of many adult Lepidoptera, even when fed as adults (e.g. Ziegler, 1991). Unfed moths lost more mass than fed moths (0.34±0.02 g *versus* 0.11±0.02 g, respectively), older moths lost more mass than did younger moths (0.26±0.02 g *versus* 0.18±0.02 g, respectively), and males lost more mass than did females (0.32±0.02 g *versus* 0.12±0.02 g, respectively). Moths that were heavier at eclosion lost more mass than did smaller moths; this trend was significant when mass loss was quantified either as a difference (Table 4) or as a percentage of eclosion mass ($F_{1,38}$=61.60, P<0.0001).

Nectar intake and its effect on respiratory quotient in fed moths

Pre-flight meal sizes among fed moths did not vary between males and females, but did decrease significantly with age (two-factor ANOVA, $F_{1,46}$=18.69, P<0.0001). Variation in the amount of nectar consumed before a flight had no effect on flight duration, respiratory quotient or rate of oxygen consumption. However, intake amounts allow a useful comparison between energy intake immediately before and energy expenditure during a flight, providing insight into whether moths have excess dietary carbohydrate available to use as fuel.

The energy potentially available from pre-flight nectar intake was estimated, assuming 72 mol ATP mol^{-1} sucrose (the ATP produced from complete carbohydrate oxidation; Withers, 1992) and the following upper and lower boundaries

Table 4. *Effects of feeding treatment, sex, age and mass at eclosion on moth mass loss (mass at eclosion minus mass at end of flight), tested using analysis of covariance*

Effect	SS	d.f.[1]	*F* ratio	*P*
Feeding treatment	0.150	1	58.00	<0.0001
Sex	0.099	1	38.30	<0.0001
Age	0.033	1	12.68	0.0011
Mass at eclosion[2]	0.253	1	97.68	<0.0001
Error	0.0853	33		

Interaction terms were non-significant and were omitted from the final analysis.

[1]*N*=38 moths.

[2]Using percentage mass loss as the dependent variable instead of absolute mass loss did not change the significance of the eclosion mass effect.

SS, sum of squares; d.f., degrees of freedom.

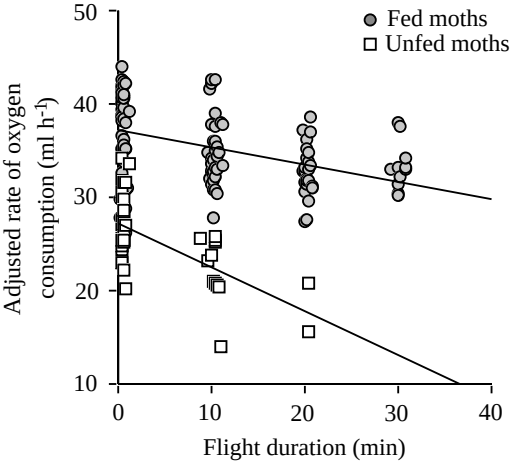

Fig. 5. Decline in adjusted rate of oxygen consumption across a flight in fed (shaded circles) and unfed (open squares) moths, indicated by the significant flight duration × feeding treatment interaction from the oxygen consumption rate ANCOVA model. Values consist of data with the effects of sex, moth identity and age removed. This allows the effect of flight duration (a continuous covariate) on rate of oxygen consumption to be visualized directly while controlling for variation introduced by the other factors in the model.

of absorption efficiency. Mean apparent sucrose absorption in *Manduca sexta* fed 30 % sucrose solution was 76 %, but exhibited high variation (R. D. Stevenson, personal communication). Apparent sucrose absorption {([$sucrose_{food}$] − [$sucrose_{feces}$])/[$sucrose_{food}$]} is likely to be an underestimate because it does not account for water absorption from ingested nectar. Here, I assume both 100 % and 70 % sucrose absorption to generate a reasonable range of ATP that would be available if the sugar meal were metabolized directly and completely.

The amount of ATP consumed during a flight can be estimated from the rate of oxygen consumption and the respiratory quotient. Rates of oxygen consumption ($\dot{V}_{O_2}$; $ml\,O_2\,h^{-1}$) are converted to $\mu mol\,h^{-1}$ ($\dot{M}_{O_2}$) using $31.6\,\mu mol\,O_2\,ml^{-1}\,O_2$ as a conversion factor. I calculated the conversion from the ideal gas law at mean laboratory temperature and pressure, which varied little over the course of the experiment (introducing less than 1 % potential variation into the conversion factor). Using RQ to obtain the fraction of each fuel burned during each flight, $\mu mol\,O_2\,min^{-1}$ was converted to $\mu mol\,ATP\,min^{-1}$, assuming that carbohydrate use yields 6 ATP molecules per O_2 molecule consumed; fat use consumes 5 ATP per O_2. Multiplying by the time at last sample gives an estimate of the total ATP consumed during the flight.

ATP available from the pre-flight sugar meal exceeded ATP consumed during the ensuing flight in almost all cases (Fig. 6). Even if we assume that only 70 % of ingested sugar is absorbed, sugar intake still exceeds consumption in most cases (Fig. 6) and by as much as fivefold. This result suggests that moths are not constrained to burn a mixture of fat and carbohydrate during flight by insufficient carbohydrates; rather, that other factors govern the mixture of metabolic substrates used during flight. The calculation is conservative in that it does not take into account endogenous sources of carbohydrate; moths fed twice a day are also likely to have stored glycogen in the flight muscle (D. M. O'Brien, unpublished observations; Stevenson, 1968; Burkhardt and Wegener, 1994) as well as in the fat body (Becker et al., 1996). If the availability of dietary carbohydrates were a constraint upon fuel use, one would expect to see a relationship between RQ and the residuals around the lines of equality in Fig. 6. Residual variation around the lines $y=x$ and $y=0.7x$ was uncorrelated with RQ; therefore, those moths that consumed too little nectar to support the ensuing flight using carbohydrate alone did not use significantly more fat during flight than those with a surfeit of carbohydrates.

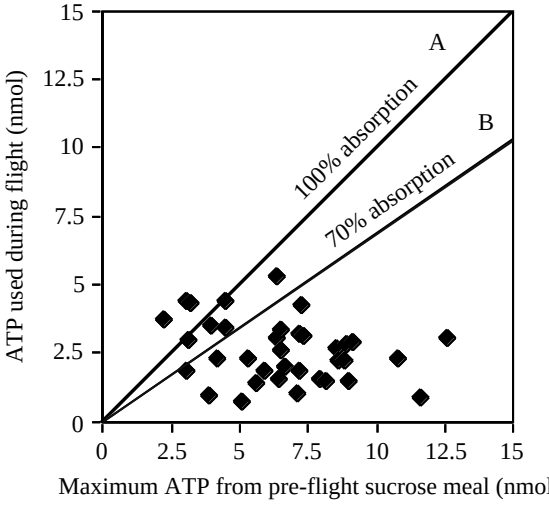

Fig. 6. Energy expenditure during flight (in nmol ATP) plotted against energy intake as sucrose before flight (in nmol ATP, the ATP that would have been generated had the entire meal been catabolized as sugar). A is the line of equality if 100 % sucrose absorption and utilization are assumed ($y=x$), whereas B is the line of equality if 70 % sucrose absorption and utilization are assumed ($y=0.7x$). Each symbol is for a separate individual.

Discussion

Importance of dietary carbohydrate as a flight fuel in Amphion floridensis

This study demonstrates that either carbohydrate or fat can serve as the primary flight fuel for the hawkmoth *Amphion floridensis* and that carbohydrate is the principal flight fuel for moths with a steady supply of dietary nectar. Given that the conversion of carbohydrates to fats is energetically expensive, it should not be surprising that an actively nectar-foraging moth is capable of metabolizing sugar directly during flight. In contrast, fat was the predominant (and usually exclusive) flight fuel among unfed moths. Fat reliance in unfed moths was also expected, given that unfed moths must support flight wholly from stored reserves. Physiological interpretation of unusually high or low respiratory quotients has complicated some studies (Chaui-Berlinck and Bicudo, 1995; Walsberg and Wolf, 1995). However, in the present study, all measurements fell precisely within the range expected for carbohydrate or fat metabolism and are therefore straightforward to interpret.

This study is the first to use respirometry to assess fuel use in a flying moth since Zebe (1954). Although the biochemical capacity for carbohydrate use in Lepidoptera has been evident for decades (Stevenson, 1968; Beenakkers, 1969; Crabtree and Newsholme, 1972; Hansford and Johnson, 1976), the present study provides the first direct evidence of carbohydrate reliance in flight. Studies that have either suggested or demonstrated the exclusive oxidation of fat during flight (e.g. Zebe, 1954; Brown and Chippendale, 1974; Ziegler and Schulz, 1986) have been widely cited as general to all Lepidoptera. By demonstrating that fuel use varies according to diet within a single species, these results highlight the danger of generalizing across all species the fuel use observed in a single study.

Carbohydrates were the primary, but not exclusive, flight fuel among fed moths. Fed moths tended to burn instead a mixture of carbohydrates and fat. Discussion of fuel mixing in the literature centers primarily around temporal shifts in fuel use (e.g. Candy et al., 1997). Migratory locusts *Locusta migratoria*, for example, switch from carbohydrate to fat oxidation after beginning flight, yet continue to utilize carbohydrates at a lower rate (Van der Horst et al., 1978). However, fuel mixing in the present study does not result from

a transition from the use of carbohydrates to the use of fats: RQs were constant during flights of up to 30 min in length. Although the metabolic mechanisms that control the switch between carbohydrate and fatty acid oxidation have been investigated (Hansford and Cohen, 1978; Storey, 1980; Wegener et al., 1987), what determines stable fuel mixing at the level of the whole organism is not well understood. Because the estimated amount of ATP potentially available from pre-flight sugar intake appears to exceed the ATP requirement for most flights, failure to support flight exclusively using carbohydrate should not be interpreted as evidence for insufficient glucose availability.

Sex differences in fuel use

Males and females used fuel in a different manner. Nectar-fed females appeared to conserve carbohydrate when newly eclosed, as revealed by significantly lower RQs than in fed males on the first day after emergence. This difference may to be related to reproductive demands. Female *Amphion floridensis* emerge with only 3 % of their oocytes fully provisioned and rapidly incorporate carbon from nectar sugars into eggs (D. M. O'Brien and D. P. Schrag, in preparation). Of the carbon present in the egg, 50–60 % derives from female nectar-feeding in this species once a female has fed for several days (D. M. O'Brien and D. P. Schrag, in preparation). The nutritional demand imposed by egg provisioning may have selected for females to spare dietary carbohydrate in flight, thereby retaining more nectar sugars for reproduction. Because the females in this study were unmated and did not oviposit, oocyte provisioning might have been expected to decline fairly rapidly. Consistent with this expectation, carbohydrate use during flight by fed females increased significantly by the third day after emergence and thereafter did not differ from carbohydrate use by males.

Variation in rates of oxygen consumption

There was no correlation between respiratory quotient and rate of oxygen consumption. Such a relationship would be expected if some aspect of differing levels of activity, such as a constraint in the rate of fuel supply, required a switch to carbohydrate use at higher metabolic rates (Wegener et al., 1987; Grafe, 1997). Although carbohydrate appears to be the preferred flight fuel for insects with particularly high rates of metabolic performance (Crabtree and Newsholme, 1972; Beenakkers et al., 1975; Gmeinbauer and Crailsheim, 1993), differences in metabolic rate do not explain the variation in fuel use observed in the present study. Tethered flight in chambers is likely to underestimate the true metabolic cost of unimpeded flight (Rayner and Thomas, 1991). Had the rate of oxygen consumption and respiratory quotient been tightly linked, respiratory quotients might have been underestimated using my methods. The lack of association, however, provides further assurance that the fuel use observed in this study is representative of physiological responses in the free-living animal.

The mean rates of oxygen consumption measured in this study are comparable with those previously measured for hawkmoths using closed-chamber respirometry, despite the fact that the moths used in this study were tethered (Bartholomew and Casey, 1978). The wide variation in rates of oxygen consumption among individuals was partly due to the effects of feeding treatment; fed moths had higher rates of oxygen consumption than unfed moths. This difference increased over time, with rates of oxygen consumption decreasing even further as the duration of starvation increased in unfed moths. This decrease was disproportionately due to males, which were visibly weakened by the end of the experiment. Accurate body masses at the time of flight were only available for a subset of oxygen consumption measurements; however, among these measurements, there was no effect of body mass variation on rate of oxygen consumption. This result is surprising because Casey (1976) did find a significant relationship between body mass and rate of oxygen consumption within *Manduca* and *Hyles* species; however, the range of body masses observed in the study of Casey (1976) was five times greater than that of the hawkmoths studied here. The lack of mass-dependence indicates that variation among individuals in rate of oxygen consumption depends directly on feeding, age and sex, rather than secondarily through their effects on body mass.

A potential explanation for increasing rates of oxygen consumption with age in fed males and females may involve changes in wing loading due to wing wear. Wing loading (wing area/body mass) is correlated with the rate of oxygen consumption in hawkmoths (Casey, 1976). Wing area in the present study was observed to decrease markedly across 5 days from abrasion on the cage mesh, although it was not measured quantitatively. Wing loading would therefore increase with age as long as the proportional loss of wing area was greater than the proportional loss of body mass. Fed moths lost on average 16 % of their body mass over the course of the experiment. The wing wear appeared to be easily greater than 40 % of total wing area, which would produce a net increase in wing loading. Because wing wear with age is widely observed in butterflies and moths in the field, the pattern of increasing rate of oxygen consumption with age may be a general trend in nature. However, further measurements are required to evaluate this potential explanation.

Implication for comparative studies of insect metabolism

The flexibility with which *A. floridensis* uses either fat or carbohydrate during flight has implications for the patterns of fuel use that have emerged from comparative studies of flying insects. Our understanding of fuel use is based on relatively few taxa and has suggested generalities that may not be correct. *Locusta migratoria* is one example: its pattern of transitory carbohydrate use giving way to lipid metabolism, although quite well studied, may be a less suitable model of metabolism for non-migratory locusts (Karhuize, 1972). Hawkmoths have been characterized as having little capacity for carbohydrate metabolism on the basis of measurements from the non-feeding poplar sphinx *Lathoe populi* (Crabtree and Newsholme, 1972)

and unfed *Manduca sexta* (Ziegler and Schultz, 1986). When nectar-feeding, however, *Amphion floridensis* relies primarily on incoming dietary carbohydrates to fuel flight costs. These observations suggest that an insect's life history, diet and foraging habit may play a critical role in determining flight fuel and emphasize the importance of understanding variability within species when characterizing patterns of fuel use among insects.

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