

Honeybee flight metabolic rate: does it depend upon air temperature?

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Summary

Differing conclusions have been reached as to how or whether varying heat production has a thermoregulatory function in flying honeybees *Apis mellifera*. We investigated the effects of air temperature on flight metabolic rate, water loss, wingbeat frequency, body segment temperatures and behavior of honeybees flying in transparent containment outdoors. For periods of voluntary, uninterrupted, self-sustaining flight, metabolic rate was independent of air temperature between 19 and 37°C. Thorax temperatures (T_{th}) were very stable, with a slope of thorax temperature on air temperature of 0.18. Evaporative heat loss increased from 51 mW g⁻¹ at 25°C to 158 mW g⁻¹ at 37°C and appeared to account for head and abdomen temperature excess falling sharply over the same air temperature range. As air temperature increased from 19 to 37°C, wingbeat frequency showed a slight but significant increase, and metabolic expenditure per wingbeat showed a corresponding slight but significant decrease. Bees spent an average of 52% of the measurement period in flight, with 19 of 78 bees sustaining

uninterrupted voluntary flight for periods of >1 min. The fraction of time spent flying declined as air temperature increased. As the fraction of time spent flying decreased, the slope of metabolic rate on air temperature became more steeply negative, and was significant for bees flying less than 80% of the time. In a separate experiment, there was a significant inverse relationship of metabolic rate and air temperature for bees requiring frequent or constant agitation to remain airborne, but no dependence for bees that flew with little or no agitation; bees were less likely to require agitation during outdoor than indoor measurements. A recent hypothesis explaining differences between studies in the slope of flight metabolic rate on air temperature in terms of differences in metabolic capacity and thorax temperature is supported for honeybees in voluntary flight, but not under agitation.

Key words: *Apis mellifera*, thermoregulation, flight energetics, water loss, wingbeat frequency, bee.

Introduction

Honeybees, like other large (>50–200 mg) insects of several orders, maintain high mechanical power production during flight by regulating thorax temperature (T_{th}) within a narrow range, often well above ambient temperatures (chapter 1 in Heinrich, 1993; Dudley, 2000). T_{th} is elevated before flying by shivering the wing muscles, and is kept high during flight as a byproduct of flight muscle activity (Heinrich, 1993; Dudley, 2000). T_{th} stabilization in many taxa involves regulation of heat loss, often by varying hemolymph flow to the abdomen, which serves as a radiator in some moths, dragonflies and bumblebees (Heinrich, 1993) or, in the case of honeybees, by regulating hemolymph flow to the head, with heat loss at higher air temperatures (T_a) augmented by evaporation of regurgitated nectar (Heinrich, 1980a,b; Cooper et al., 1985).

In theory, insects could maintain thermal stability in flight by regulating heat gain, heat loss, or both. Under shaded conditions, where short-wave radiative heat gain is small, metabolic heat production is the primary source of heat gain (Cooper et al., 1985; Coelho, 1991; Roberts and Harrison,

1999), but its role in maintaining thermal balance over a range of T_a values has been debated. Initial measurements of honeybees found no association between flight metabolic rate (FMR) and T_a (Heinrich, 1980b), and flying honeybee heat exchange models published in the subsequent decade accepted this as a key assumption (Cooper et al., 1985; Coelho, 1991). This was in agreement with conclusions reached for other insect orders, where FMR was associated with mechanical flight requirements (Casey, 1989; Wolf et al., 1989; Heinrich, 1993), and thermal stability was maintained at higher T_a values through various mechanisms for regulating heat loss (reviewed in Heinrich 1981, 1993; Dudley, 2000). More recent studies, though, have concluded that flying honeybees, as well as other bees and the dragonfly *Anax junius*, do indeed maintain thermal stability, at least partly by varying heat production (May, 1995; Harrison et al., 1996a,b; Roberts et al., 1998; Roberts and Harrison, 1999; Borrell and Medeiros, 2004; reviewed for bees in Roberts and Harrison, 1998; for honeybees, Harrison and Fewell, 2002), and other related

research has supported this mechanism, though with caution (Moffatt, 2001). In studies reporting wingbeat frequency (WBF) as well as FMR, both variables show generally similar relationships with T_a , and measurements of WBF have been presented as corroborative evidence of T_a effects on FMR (May, 1995; Harrison et al., 1996b; Roberts et al., 1998; Borrell and Medeiros, 2004). Evidence that load and FMR in honeybees may be decoupled (Balderrama et al., 1992; Moffatt, 2000) or only weakly associated (Feuerbacher et al., 2003) suggest that varying heat production during flight is a plausible mechanism for maintaining thermal balance during flight in nature. A recently proposed explanation (Harrison and Fewell, 2002) arises from the observation that force production of honeybees in tethered flight reaches a peak value when T_{th} is about 38°C and declines as T_{th} increases or decreases (Coelho, 1991). Harrison and Fewell (2002) hypothesize that FMR may follow a similar pattern, with the result that the range of measurement T_a values chosen, along with differences in bees' metabolic capacity between studies, may explain differing conclusions about the role of varying heat production in maintaining thermal stability during flight.

Another possible explanation is differences in experimental conditions. Making precise measurements of FMR of insects requires subjecting animals to conditions different from those encountered in nature, and the consequences of these differences are not always obvious. While open-flow respirometry offers the advantages of high accuracy and temporal resolution, it requires that subjects maintain free flight within a confined space, in the absence of natural referents, for a period of time sufficient to yield a stable signal. Researchers have employed a variety of tactics to elicit flight under confinement or restraint; for bees, these include visual cues (Esch, 1976; Esch et al., 1975; Wolf et al., 1989; Jungman et al., 1989; Nachtigall et al., 1989; Feller and Nachtigall, 1989; Ellington et al., 1990; Hrassnig and Crailsheim, 1999; Feuerbacher et al., 2003), tarsectomy, amounting to landing gear removal (Heinrich, 1980b), tethering (Sotavalta, 1954; Esch et al., 1975; Esch, 1976; Jungmann et al., 1989; Feller and Nachtigall, 1989; Nachtigall et al., 1989; Coelho, 1991; Hrassnig and Crailsheim, 1999) and flight chamber motion, using either constant agitation (Harrison and Hall, 1993; Harrison et al., 1996a,b, 2001; Harrison and Fewell, 2002) or occasional and minimal shaking of the chamber (Heinrich, 1980b; Roberts et al., 1998; Roberts and Harrison, 1999). For flying honeybees, the association between FMR and T_a has, under different measurement conditions, variously been found negative (Harrison et al., 1996a,b; Roberts and Harrison, 1999), positive (Hrassnig and Crailsheim, 1999; Harrison et al., 2001), or not significant (Heinrich, 1980b). Nevertheless the honeybee, with its capacity for hovering and its relative willingness to fly in confinement, is a system of choice for addressing flight energetics questions, and FMR has been measured more often for the honeybee than for any other animal (Harrison and Fewell, 2002).

We re-examined whether, for honeybees flying in

confinement, variation in FMR contributes to the maintenance of thermal stability. Our choice of an outdoor location was driven by early trials, which we report, that showed greater willingness to sustain flight in a respirometry chamber outdoors than under otherwise similar conditions indoors. We sought to explain previous differing conclusions by performing two experiments that, in combination, cover much of the methodological ground of previous work while quantifying the voluntary flight and non-flight behavior of bees flying in small chambers under largely natural outdoor lighting conditions, accounting for all bees measured. Specifically, we asked: (1) what is the relationship between FMR and T_a for honeybees in continuous, unprovoked, self-supporting flight? (2) What is the relationship between T_{th} and T_a ? (3) What is the contribution of evaporative heat loss to body temperature stability? (4) Do wingbeat frequency (WBF) and FMR have similar relationships with T_a ? (5) Does agitation of the flight chamber or incidence of non-flight behavior affect the relationship of FMR and T_a ? We then examined the possibility that differences in T_{th} could explain differing conclusions about the relationship of FMR and T_a between studies.

Materials and methods

Animals and measurement conditions

Honeybees *Apis mellifera* L. were captured from a single colony maintained by B. H. in Hinesburg, VT, USA, during August 28–30 and September 12–14, 2003, between 8:00 h AM and 16:30 h EST. Outgoing foragers and guards were grasped by leg at the hive entrance with tweezers and placed in a 35 mm film canister, then transferred without further handling to the respirometry chamber within less than 1 min. 5 min measurements were thus completed within less than 6 min of capture.

The respirometry chamber, a 500 cc Pyrex Erlenmeyer flask, was housed in a transparent temperature control cabinet placed in a shaded outdoor location. The 0.38 m high \times 0.31 m diameter temperature control cabinet consisted of a 0.64 cm Plexiglas top and bottom, secured by a wooden frame, with a sheet of 0.2 mm acetate forming a cylinder comprising the side wall; access to the chamber was through the overlapping edges of the acetate sheet. Temperature was raised by means of a warm air blower whose output was ducted by 3.2 cm PVC tubing through a fitting in the floor of the cabinet and directed away from the respirometry chamber; a 5 cm aperture in the top Plexiglas panel of the cabinet served as an exhaust. Chamber T_a values ranging from 18 to 39°C were maintained to within $\pm 0.5^\circ\text{C}$. Measurements made at a given T_a value were distributed across different times of day so that T_a treatment was independent of both time of day ($P=0.71$) and the number of hours before or after solar noon ($P=0.75$).

If a bee ceased flying during measurement, the chamber was picked up by the lip within 0.5 min, tapped 3–4 times or shaken lightly and then set down. This procedure was repeated at approximately 0.5 min intervals if the bee did not resume flight; thus, brief chamber motion sometimes caused bees to

initiate flight, but was not used to sustain flight in bees that persisted in landing.

Respirometry, body segment temperature and wingbeat frequency measurement

Carbon dioxide production and water loss were measured by differential open-flow respirometry. Air scrubbed of water and carbon dioxide by soda lime-Drierite-soda lime columns was flowed at 860 cc min^{-1} through the respirometer chamber. Bev-A-Line tubing (Thermoplastic Processes Inc., Georgetown, DE, USA) was used throughout except between scrubber columns. T_a inside the chamber was monitored to $\pm 0.1^\circ\text{C}$ using a Physitemp BAT 12 field thermometer (Physitemp Instruments, Clifton, NJ, USA) with its sensor inside a hypodermic needle inserted through the chamber stopper. Data were digitized using a converter (ADC-1, Remote Measurement Systems, Seattle, WA, USA) and recorded using a computer and Datacan V software (Sable Systems, Henderson, NV, USA). Flight activity and wingbeat frequency were recorded on a standard cassette recorder (Radio Shack CTR 123) whose microphone (Realistic catalog no. 331052) was inserted through the chamber stopper. The same recorder was used for both recording and playback, and was powered by an external AC power supply throughout to ensure uniform tape speed. Carbon dioxide production and water loss were measured using a Li-Cor 6262 analyzer (Li-Cor, Lincoln, NE, USA), using 0.1 min signal averaging, with signals recorded every 0.1 min. Corrections for dilution and infrared band-broadening arising from the inclusion of subjects' water loss in the excurrent air column were made by the Li-Cor unit. Each data point presented represents the mean for the measurement period. Data from the first minute of measurement were not used except for measurements when chamber temperature was not elevated above ambient; 1 min of equilibration is sufficient for honeybees to reach thermal equilibrium even at elevated T_a (Roberts and Harrison, 1999). The chamber was kept in the temperature control cabinet and flushed with scrubbed air between measurements.

At the conclusion of respirometry measurement, the chamber stopper was removed and a plastic bag that had been kept within the temperature control cabinet was immediately placed over the mouth of the respirometry chamber. The bag and chamber were then removed from the cabinet and the bee was shaken into the bag and restrained against a sheet of foam as rapidly as possible; within 10 s, thorax (T_{th}), then head (T_h), then abdomen (T_{ab}) temperatures were measured using a hypodermic needle thermocouple probe (Heinrich, 1993) connected to a Physitemp BAT 12 field thermometer. Body segment temperatures are reported only for bees that were flying for the final 30 s of respirometry measurement and that had sustained flight for >40 s of the final min of measurement. Body and nectar crop content mass were determined immediately after measurements to the nearest 0.001 g using a Sartorius L4205+ pan balance (Sartorius GMBH, Göttingen, Germany).

Analysis

Respirometry data were transformed and analyzed using Datacan V, with washout correction performed as described in Bartholomew et al. (1981), as implemented in Datacan V; statistics were done in SPSS 11 for Macintosh (SPSS Inc, Chicago, IL, USA). CO_2 production values were converted to W assuming carbohydrate metabolism (Beenakkers et al., 1984). Early observations that WBF of some bees declined audibly during the measurement period made it clear that post-respirometry WBF measurements would not be representative. However, the air pump and blower motor contributed background noise during respirometry, making analysis of digitized recordings made during respirometry problematical. We therefore determined WBF for each 0.5 min of respirometry measurement from the pitch of the flight tone (Sotavalta, 1947). Analysis was performed by one of us (W.A.W.) who has absolute pitch, as did Sotavalta (1947), but using a keyboard verified to be tuned to concert pitch ($A=440 \text{ Hz}$) as a reference. Recordings were sampled continuously and estimated pitch noted at least twice during each 0.5 min interval, and more frequently if pitch varied. In a double-blind verification of this method, $9 \times 30 \text{ s}$ recording sections that did not include blower motor noise were re-analyzed by M. Schindlinger using Cool Edit software (Syntrillium/Adobe Systems, San Jose, CA, USA); acoustically determined values averaged $2.0 \pm 3.5 \text{ Hz}$ (mean \pm S.E.M.), or 1.1%, higher than digitally determined values, a difference that was not significant (paired-sample t -test, d.f.=8, $P>0.1$). Periods of flight were timed from recordings by stopwatch, with very brief buzzing intervals of $<1 \text{ s}$ omitted as not representing flight; these measurements were repeatable to within less than 3%. To synchronize sound recordings with respirometry traces, a time base correction of -12 s was applied to recordings to account for the 9 s respirometry system time delay (established by bolus injection using a 1 s sampling interval) plus a 3 s delay arising from our 6 s signal averaging period. Periods of self-sustaining flight with no interruption or provocation for at least 1 min, and which did not include periods of substantial ($>10\%$) decline in WBF, were termed first quality flight and are reported as a subset of our measurements.

Head and abdomen temperature excess ratios (R_h and R_{ab} , respectively) are the ratios of head or abdomen temperature excess to thorax temperature excess, calculated as $R_h=(T_h-T_a)/(T_{th}-T_a)$ and $R_{ab}=(T_{ab}-T_a)/(T_{th}-T_a)$; these values are predicted to be independent of T_a if heat transfer between segments does not change, but to be associated with T_a if thermoregulation involves changes in heat transfer between body segments. For example, an increase in the value of R_h or R_{ab} at higher values of T_a would indicate an increased shunting of heat to the head or abdomen, suggesting use as a thermal window to dissipate thorax heat, at least in the absence of changes in other pathways of heat exchange (Baird, 1986; Stavenga et al., 1993; Roberts and Harrison, 1999). A decrease to negative values in R_h or R_{ab} at higher T_a would indicate evaporative cooling from the head or abdomen, respectively (Roberts and Harrison, 1999).

Since no pyranometer was available, we accounted for solar short-wave radiative heat gain by making hourly measurements with a microeinstein meter (Li-Cor), with its sensor pointed directly upwards in full sun, and both upwards and downwards in the shade. Full sun values were indexed to direct solar radiation in W m^{-2} as calculated from the solar zenith angle for the dates and latitude of our measurements (appendix B in Stevenson, 1985). The ratios of the microeinstein values in shade to those in full sun were used to establish the fraction of full sun values encountered in our shaded location. Honeybee surface area was estimated by scaling values reported by Roberts and Harrison (1999) to the mean body mass of our bees, assuming a surface area mass scaling exponent of $2/3$. Calculated short-wave radiation from overhead and from below in shade were each assumed to strike 50% of the bee's surface area (Kenagy and Stevenson, 1982; Cooper et al., 1985).

Effect of agitation

In a separate set of measurements, we sought to determine whether the degree of flight chamber agitation required to maintain flight affected the association between FMR and T_a . Bees were captured and handled as described previously, but during a different year and from a different colony maintained by B. H. in Hinesburg, VT, USA, between 08:30 h and 17:30 h during late July and early August. Respirometry was done as previously described except that we used Tygon tubing (Saint-Gobain Performance Plastics, Bridgewater, NJ, USA), since its elasticity assured secure seals at fittings when the chamber was being vigorously shaken. Because of Tygon tubing's hygroscopic properties (J. R. B. Lighton, personal communication), temporal response for water loss is much slower and the values less trustworthy; we therefore do not report water loss data for this experiment. No temperature control cabinet was used; instead, chamber T_a was allowed to track ambient temperature in shade. WBF was not recorded. Bees were kept airborne by administering chamber agitation only when they attempted to land. Behavior, as recorded in field notes and by markers in the Datacan V files, was divided into three categories. In the first, which we term 'non-agitated flight', bees flew with few or no landing attempts (not extending their legs or changing body angle in apparent preparation for landing). In the second, 'agitated flight', bees repeatedly attempted to land and were kept in the air by frequent or constant chamber agitation. In the third, 'intermediate flight', behavior was varied or intermediate, with no portion of the measurement clearly representing either specific behavior. Respirometry data analysis was as in the primary experiment.

Results

First-quality flight

Of the 78 honeybees measured, 19 displayed first-quality flight, i.e. periods of 1 min or more of uninterrupted, self-supporting flight without provocation and with steady WBF

values. FMR values for periods of steady WBF decline of $>10\%$ were not considered representative of first-quality flight (Fig. 1A). We used this criterion because, of the 24 bees whose WBF declined by $>5\%$ during respirometry, only three had

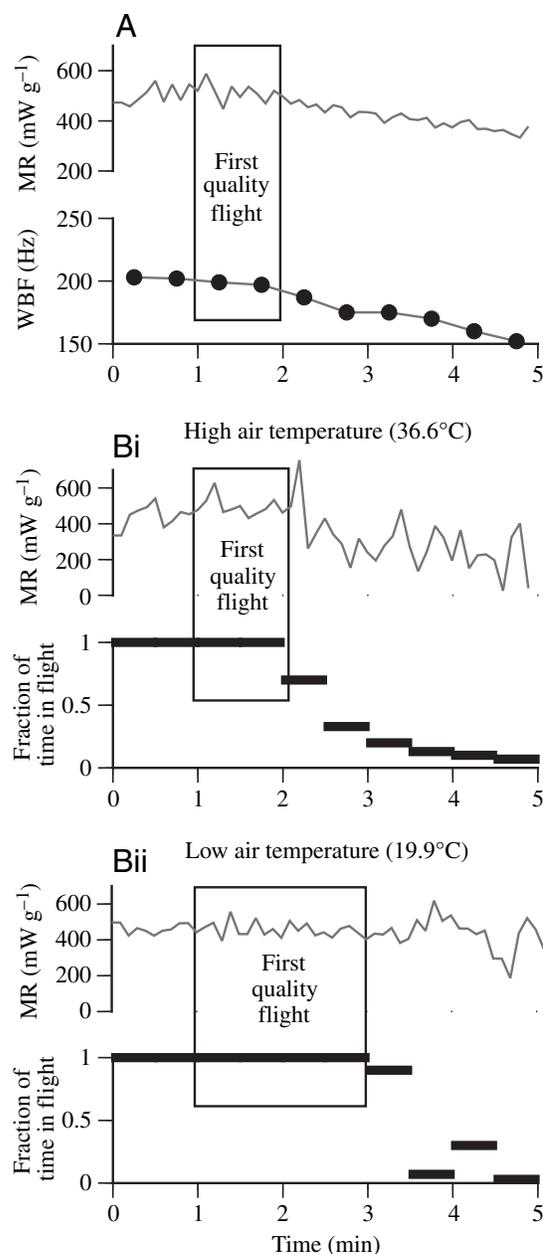


Fig. 1. Data traces from three honeybees of 78 measured in an 0.5 l glass respirometry chamber within a transparent temperature control cabinet under shaded outdoor conditions. Periods of unprovoked, uninterrupted free flight, termed first-quality flight (FQF) are indicated, as well as periods that were excluded from FQF data because of declining wingbeat frequency (WBF) or intermittent flight (B). In A, metabolic rate (MR) declined by 28% and WBF by 22% during the final 3 min of respirometry. In the higher temperature measurement (Bi), MR during the first min after flight became intermittent and was 41% lower than during FQF, and during the final min was 54% lower. In the lower temperature measurement (Bii), MR during the final 2 min was 4% lower than during FQF.

nectar present in the honeycrop, whereas 31 of the 54 bees whose WBF declined <5% had nectar present, raising the possibility that diminishing WBF resulted from depleted energy reserves and might be associated with metabolic rate and body temperature values that did not represent steady-state flight (see Figs 5, 7). For bees flying at high T_a , metabolic rate diminished if flight became intermittent or ceased altogether, while for bees flying at low T_a there was little difference in metabolic rate between periods of flight and periods of non-flight behavior (Fig. 1B).

For bees in first-quality flight, FMR was not associated with T_a (Fig. 2A). FMR was also independent of time of day ($P=0.92$) and of the number of hours before or after solar noon ($P=0.21$). There was, however, a positive relationship between WBF and T_a (Fig. 2B, $r^2=0.34$, $P=0.01$), though this rested upon measurements at T_a values between 19 and 31°C; for T_a

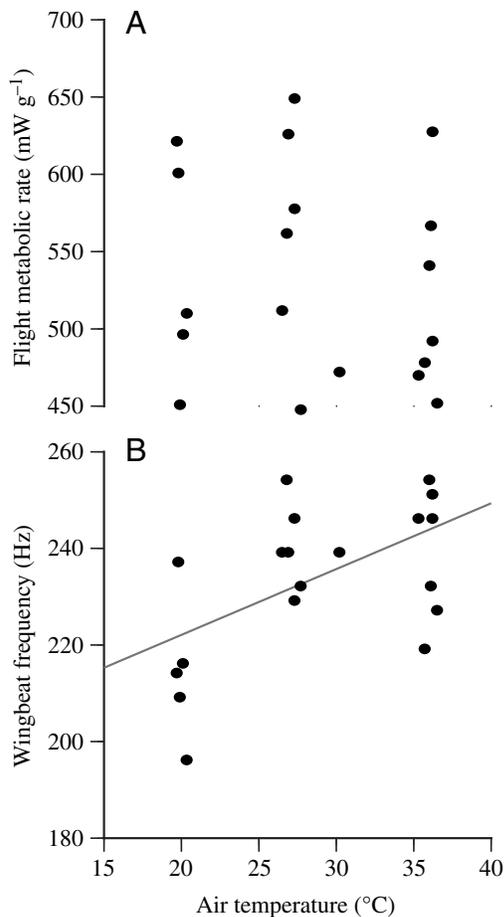


Fig. 2. (A) Flight metabolic rate (FMR) and wingbeat frequencies (WBF) vs air temperature (T_a) for periods of first-quality flight lasting for at least 1 min, for honeybees in an 0.5 l glass chamber within a transparent temperature control cabinet under shaded outdoor conditions. 19 of 78 bees measured displayed this behavior. FMR was independent of T_a ($FMR=-1.675T_a+584.9$, $N=19$, $r^2=0.03$, $P=0.51$). (B) WBF was associated with T_a between 19 and 37°C (least-squares regression shown, $WBF=1.362T_a+194.8$, $N=19$, $r^2=0.31$, $P=0.014$), but not between 26 and 37°C (regression not shown, $N=14$, $r^2<0.01$, $P=0.89$).

values between 25 and 37°C, there was no association ($N=14$, $r^2<0.01$, $P=0.89$). We did not find an association between WBF and FMR during periods of first-quality flight ($r^2=0.11$, $P=0.15$). However, we did find a negative association between metabolic energy expenditure per wingbeat and T_a (Fig. 3), amounting to a reduction of 14.4% between 20 and 37°C, though significance was marginal ($N=19$, $r^2=0.21$, $P=0.046$).

Body temperature

Body segment temperatures are reported for the 36 of 78 honeybees that were flying at the end of respirometry and had flown for more than 2/3 of the final 1 min and for the final 30 s immediately prior to body temperature measurements (Fig. 5). 13 of the 19 bees displaying 1 min or more of first-quality flight (Figs 2–4) met these criteria and are included in Fig. 5. Data for four bees whose WBF had declined by between 18 and 25% during measurement and that had empty honeycrops, suggesting energy reserve depletion, but that flew during the final minute as described, are shown but are excluded from the displayed regressions. Although T_{th} was significantly affected by T_a , the slope of the least-squares regression is relatively shallow at 0.181; bees maintained relatively stable thorax temperatures (T_{th}) of $38.5\pm 2.1^\circ\text{C}$ (mean \pm s.d., $N=31$) over T_a values ranging from 18 to 39°C. T_{th} and T_{ab} were likewise significantly affected by T_a (Fig. 5), though the relationships were distinctly nonlinear, with both T_{th} and T_{ab} falling significantly below T_a at T_a values $>34^\circ\text{C}$ (one-tailed paired-sample t -test, d.f.=9; for T_{th} , $P<0.01$; for T_{ab} , $P<0.0001$).

At T_a values $<28^\circ\text{C}$, the slopes of both the head and abdomen temperature excess ratios (R_h and R_{ab}) vs T_a were not significantly different from zero (Fig. 6A; P for each slope <0.01), while above 34°C the slopes were nearly vertical (Fig. 6A). The abrupt change in these ratios corresponded to a sharp increase in evaporative heat loss in the same T_a range (Fig. 6B).

Calculated direct solar radiation at our 44.47°N latitude at

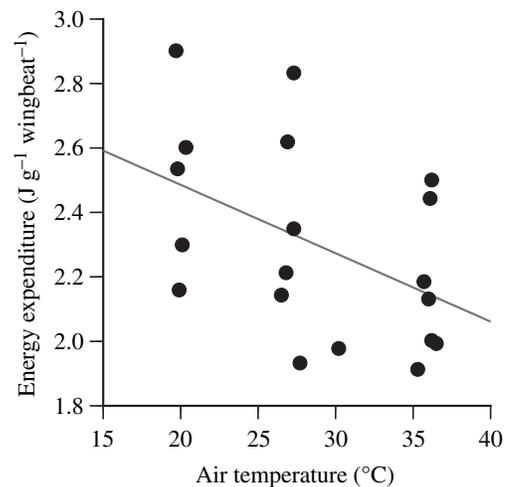


Fig. 3. For the 19 honeybees maintaining first-quality flight for 1 min or longer (Fig. 2), metabolic expenditure during each wingbeat declined as air temperature increased ($r^2=0.21$, $P=0.046$).

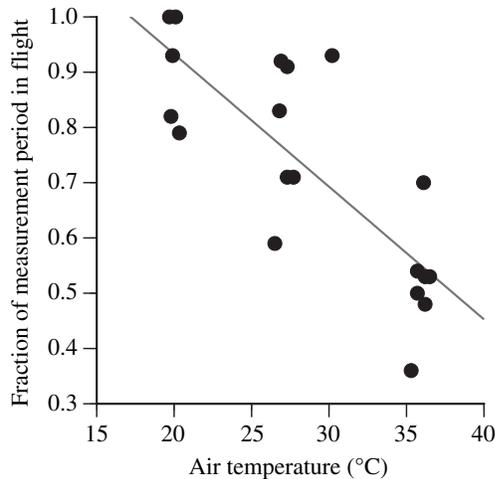


Fig. 4. For the 19 honeybees that displayed at least 1 min continuous of first-quality flight (Fig. 2), the fraction of the entire measurement period spent in flight declined as air temperature (T_a) increased ($r^2=0.63$, $P<0.0001$).

solar zenith on August 29 was 882 W m^{-2} . At solar noon under clear conditions, microeinstein values with the sensor oriented upward in the shade were 4.4% of values with the sensor similarly oriented in direct sunlight; with the sensor oriented downward in shade, values were 2.3% of those in full sunlight. Corresponding values 1 h before sunset, the approximate time of each day's final measurements, were 2.8 and 1.9% of full noon sun values. Mean calculated body surface area, based on mean body mass of $0.090 \pm 0.001 \text{ g}$ (\pm S.E.M.) was 265 mm^2 ; calculated short-wave heat gain by individual bees therefore ranged from 58 to 84 mW g^{-1} . Since T_a treatment was independent of both time of day and mean hours before or after solar noon, bees experienced a mean heat gain of about 71 mW g^{-1} across T_a treatments. Occasional broken low cloud cover (present during a minor portion of our measurements) would be expected to reduce these values by 15–30% (Monteith, 1973).

Effect of behavior

The mean fraction of the measurement period spent in flight (FTF) for the full sample of 78 honeybees was 0.52; FTF was not affected by time of day ($P=0.48$) or by the number of hours before or after solar noon ($P=0.73$). For all 78 bees, FTF showed a weak decline as T_a increased, though it was not significant ($N=78$, $r^2=0.04$, $P=0.08$). However, for the 19 bees that displayed 1 or more min of first-quality flight (Fig. 4), this relationship was strongly significant ($r^2=0.63$, $P<0.0001$).

FTF had a pronounced effect on the relationship between metabolic rate and T_a (Fig. 8). For bees that flew for 80% or more of the time, the relationship was not significant ($N=14$, $r^2=0.02$, $P=0.66$). However, for bees flying between 40 and 79% of the time, the slope of metabolic rate on T_a was steeper and strongly significant ($N=36$, $r^2=0.31$, $P<0.0001$), and became still steeper and more strongly significant for bees that flew for less than 40% of the measurement period ($N=28$, $r^2=0.60$, $P<0.00001$).

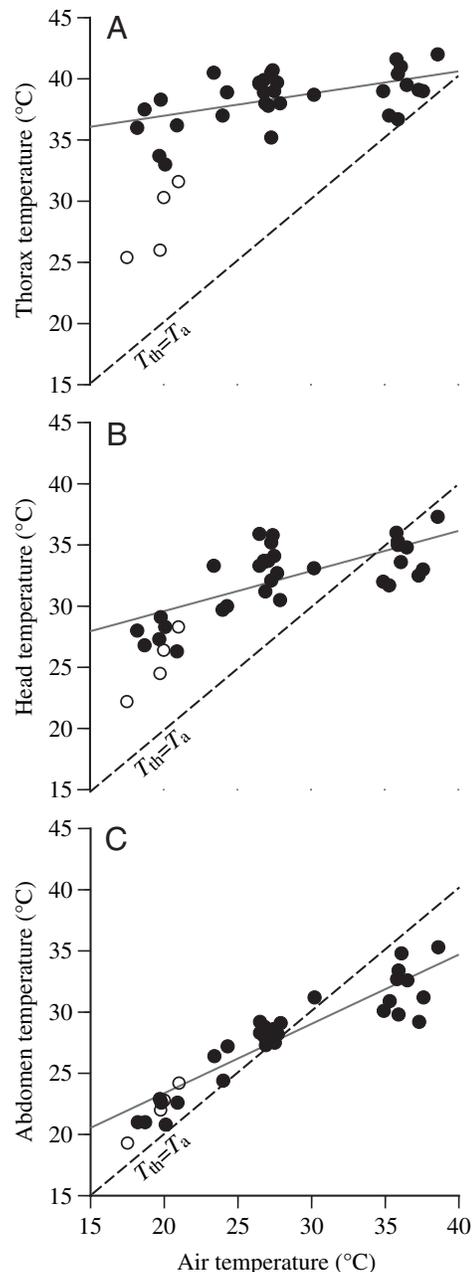


Fig. 5. Relationships of (A) thorax (T_{th}), (B) head (T_h) and (C) abdomen (T_{ab}) temperatures to air temperature (T_a) for honeybees that sustained flight for $>2/3$ of the final period of respirometry (35 of the 78 bees in Fig. 8). Four bees (open symbols) that showed the largest declines in wingbeat frequency (18–25%) during measurement are excluded from the least-squares regressions shown. Regressions: $T_{th}=0.181T_a+33.35$, $N=32$, $r^2=0.27$, $P<0.01$; $T_h=0.328T_a+23.02$, $N=32$, $r^2=0.50$, $P<0.00001$; $T_{ab}=0.566T_a+12.04$. Regressions including bees with declining wingbeat frequencies (not shown): $T_{th}=0.369T_a+27.27$, $N=36$, $r^2=0.38$, $P<0.0001$; $T_h=0.420T_a+20.04$, $N=36$, $r^2=0.58$, $P<<0.00001$; $T_{ab}=0.592T_a+11.22$, $N=36$, $r^2=0.86$, $P<<0.00001$.

For the full sample of 78 bees regardless of FTF, mean WBF over the measurement period was independent of T_a for bees whose WBF declined by $<5\%$ (Fig. 7, $N=54$, $r^2<0.01$, $P=0.69$).

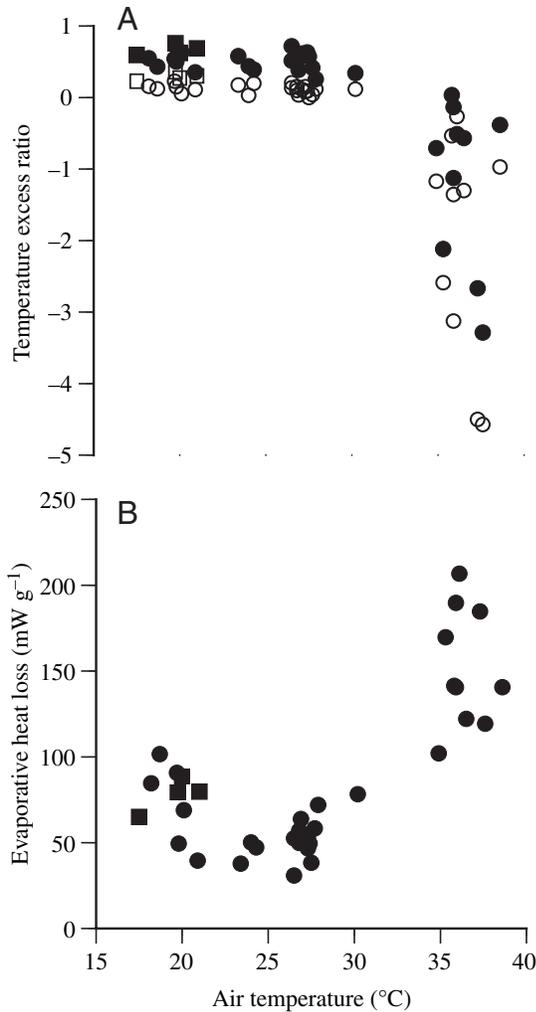


Fig. 6. (A) Relationships of head (R_h , filled symbols) and abdomen (R_{ab} , open symbols) temperature excess ratios, and with $R_h = (T_h - T_a) / (T_{th} - T_a)$ and $R_{ab} = (T_{ab} - T_a) / (T_{th} - T_a)$ to air temperature for honeybees that sustained flight for $>2/3$ of the final 1 min of respirometry. The four bees that showed the largest declines in wingbeat frequency (18–25%; see Fig. 5) are represented by squares. (B) Evaporative heat loss vs T_a for the same honeybees as in A. The four bees showing the largest declines in wingbeat frequency in A are indicated by square symbols.

Among bees whose WBF declined by $>5\%$, several at low T_a yielded the lowest WBF values we recorded; however, WBF was still independent of T_a (Fig. 7, regression not shown; $N=24$, $r^2=0.06$, $P=0.25$). FTF had little effect on this relationship, with none of the three categories showing a significant association (for 0–39% flight, $N=28$, $r^2=0.07$, $P=0.17$; for 40–79% flight, $N=36$, $r^2<0.01$, $P=0.66$; for 80–100% flight, $N=14$, $r^2=0.02$, $P=0.63$).

Effect of agitation

The response of metabolic rate to T_a differed between honeybees in agitated and non-agitated flight. For bees in non-agitated flight, metabolic rate was independent of air temperature (Fig. 2A). In contrast, for honeybees in agitated

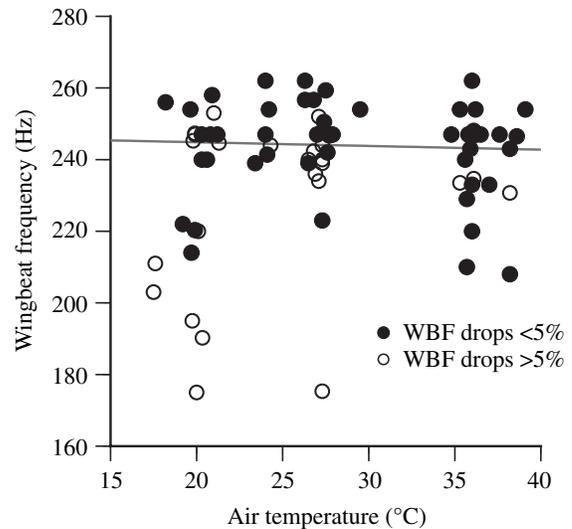


Fig. 7. For honeybees whose wingbeat frequency (WBF) declined $<5\%$ during respirometry (closed circles; 54 of the 78 bees in Fig. 8), mean WBF over the period corresponding to the averaged metabolic rate trace was independent of air temperature (T_a) (regression shown, $N=54$, $r^2<0.01$, $P=0.69$). For the 24 bees whose WBF declined by $>5\%$ (open circles), despite some low WBF values at lower T_a , the relationship was not significant (regression not shown, $N=24$, $r^2=0.06$, $P=0.25$).

flight, metabolic rate decreased as chamber air temperature increased, with values at 38°C less than two thirds of those at 22°C (Fig. 2B). Metabolic rate values at lower temperatures were similar for agitated and non-agitated fliers, while differences in values at higher temperatures accounted for the difference between the two categories (Fig. 2). After discarding data that included pronounced declines in metabolic rate, possibly indicating depletion of energy reserves, there was no relationship between mean time from the beginning of measurement to the midpoint of the trace averaged and the rate of CO_2 production ($N=59$, $r^2=0.06$, $P=0.19$).

Measurements of the first eight honeybees in this data set were completed indoors. Since only one of these bees met our criteria for non-agitated flight (Fig. 9), we moved the apparatus outdoors into a shaded location, where non-agitated flight behavior was much more frequent (30 of 51 bees), and there completed all further measurements. While collecting the data in our primary data set (Figs 1–8), we made 20 additional indoor measurements; eight of these bees displayed non-agitated flight. In all, only 32% (9) of the 28 bees measured indoors displayed non-agitated flight, compared with 59% (37) of the 51 bees measured outdoors. Because metabolic rate values in our primary data set were overall about 15% lower, raising the possibility of seasonal or colony effects (reviewed in Harrison and Fewell, 2002), these supplementary measurements are not included in the regressions in Fig. 9. However, their inclusion did not alter the conclusions for either flight quality category (for non-agitated flight, $\text{MR} = -0.903T_a + 676.3$, $N=39$, $r^2<0.01$, $P=0.78$; for agitated flight, $\text{MR} = -19.47T_a + 1122$, $N=28$, $r^2=0.52$, $P<0.0001$).

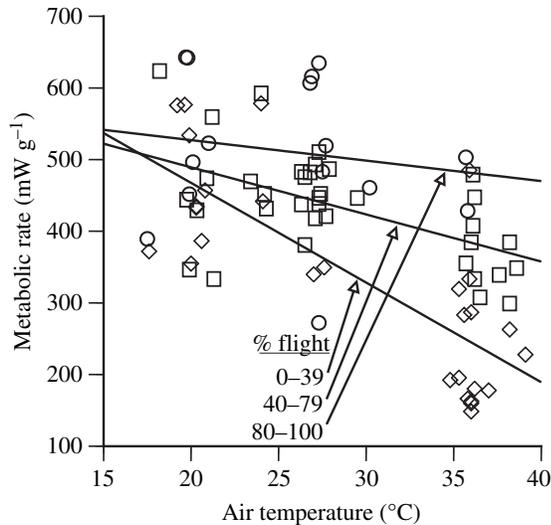


Fig. 8. For 78 honeybees including those in Figs 1–4, all measured under the same conditions, the association between metabolic rate and air temperature weakened as the fraction of time spent in flight increased [least-squares regressions: for 0–39% flight (circles), $N=28$, $r^2=0.60$, $P<0.00001$; for 40–79% flight (squares), $N=36$, $r^2=0.31$, $P<0.001$; for 80–100% flight (triangles), $N=15$, $r^2=0.02$, $P=0.58$].

Discussion

Metabolic rate in first-quality flight

Multiple relationships have been found between FMR and T_a for flying honeybees. In the present study FMR was independent of T_a , not only for first-quality flight (Fig. 2) but also for all bees that maintained flight for 80% or more of the full measurement period (Fig. 8). Of four comparable reports examining this relationship for untethered honeybees, two found an inverse relationship between FMR and T_a (Harrison et al., 1996b; Roberts and Harrison, 1999), one found a positive relationship (Harrison et al., 2001) while one found FMR independent of T_a (Heinrich, 1980b) (Fig. 10). Most (though not all) of the data in Harrison et al. (1996) were for bees under constant agitation, and the possibility was raised that this might have contributed to the result (Heinrich and Esch, 1997; Stevenson and Woods, 1997). However, the Heinrich (1980b) and Roberts and Harrison (1999) studies reached their differing conclusions even though both employed only minimal agitation in response to occasional brief cessation of flight, and both discarded data for bees displaying poor flight (J. Harrison and B. Heinrich, personal communication).

What could account for these differing outcomes? Harrison and Fewell (2002) have hypothesized that a combination of two factors may provide an explanation. First, honeybees in the different studies may have had different metabolic capacities. Such variation has been attributed to differences between colonies (Harrison et al., 1996b), genotypes (Harrison and Hall, 1993; Harrison et al., 1996b), seasons (Harrison et al., 2001) and foraging task (Feuerbacher et al., 2003). Second, FMR and T_{th} may be positively correlated below a particular T_{th} and negatively correlated above it; Harrison and Fewell

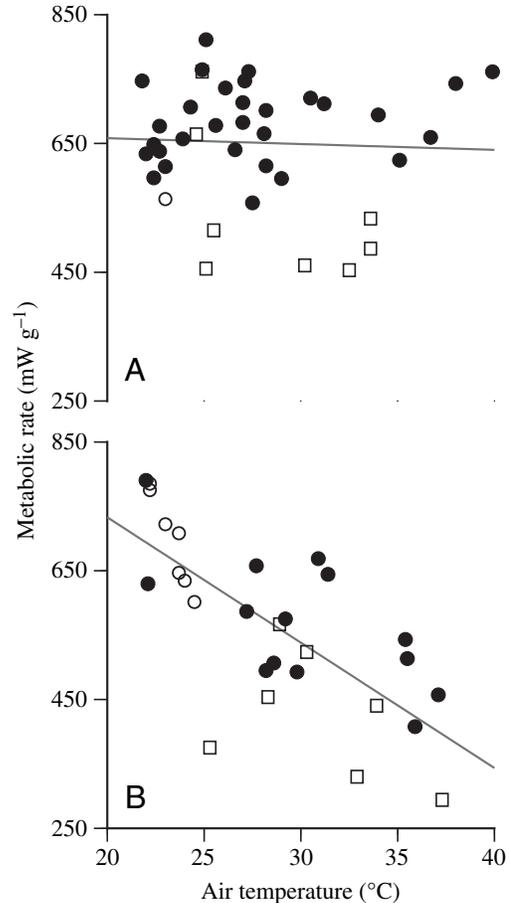


Fig. 9. The response of metabolic rate (MR) to air temperature (T_a) for a separate sample of 52 honeybees flying in an 0.5 l glass chamber, with chamber agitation administered as needed to keep bees airborne. Closed symbols denote measurements made outdoors under shaded conditions; open symbols denote indoor measurements. Square symbols represent additional indoor measurements from a different time of year, and are not included in the regressions. (A) For bees that maintained flight and made few or no landing attempts with little or no chamber agitation, MR was independent of T_a ($N=31$, $r^2=0.05$, $P=0.23$). (B) For bees that made repeated landing attempts and were kept airborne by chamber agitation, MR was inversely associated with T_a ($N=21$, $r^2=0.61$, $P<0.00001$).

(2002) propose that a T_{th} of 38°C, at which Coelho (1991) found maximum flight force production for tethered honeybees (Fig. 11A), might correspond to a maximum value for FMR, with FMR decreasing as T_{th} either increased or decreased.

The relationship between flight force production (FFP) and FMR in free-flying honeybees has not been directly tested. However, for our and other studies of honeybees, FMR during self-supporting flight and FFP during tethered flight show similar patterns of response to T_{th} (Fig. 11). As T_a increases to 47°C, both FMR and FFP fall to the minimum values associated with flight (Coelho, 1991; Harrison and Fewell, 2002), and the ratio of minimum values for flight to maximum values attained is similar for FMR and FFP (Fig. 11). The effect of T_a on FMR for hovering or slow forward flight has

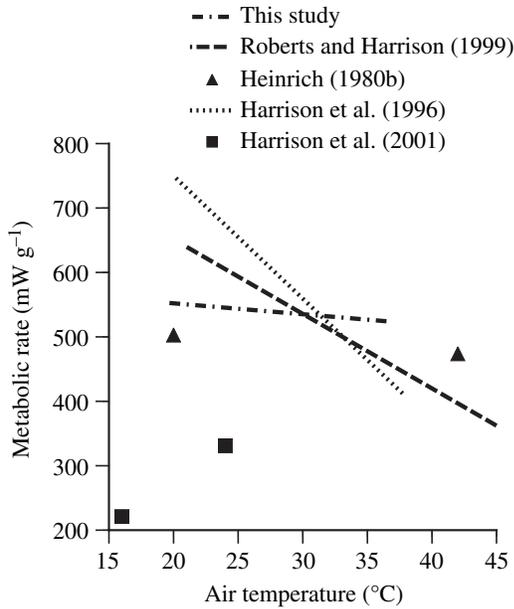


Fig. 10. The relationships of metabolic rate and air temperature for five studies of honeybees. The present study plus those of Roberts and Harrison (1999) and Heinrich (1980b) are of wholly or largely voluntary flight; those of Harrison et al. (1996, 2001) are of bees under constant agitation. Traces denote regressions for studies that include measurements at intermediate temperatures; data points denote mean values for studies not reporting measurements at intermediate temperatures. Metabolic rate is independent of air temperature only in the present study and in Heinrich (1980b). The data from Harrison et al. (2001) are for bees removed from the hive during winter.

been measured only for air temperature values that yielded relationships that were nominally linear and were so reported; accordingly, the range of air temperatures measured in each study, together with between-study differences in the bees' capacity for heat production and loss at a given T_a , can determine slope of FMR on T_a by shifting the endpoints to the left or right along what may be an overall nonlinear function (Fig. 11). Examining FMR and T_a for discrete T_a ranges in each study suggests an even closer relationship between FMR and FFP than do the reported linear relationships of FMR and T_a (Fig. 11). In our study, independence of FMR and T_a may therefore be explained by the relatively narrow T_{th} range, the bees defended, all falling within the 36–41.5°C range, for which Coelho (1991) found that FFP did not fall below 95% of the maximum value reached at 38°C. Similarly, the inverse association between FMR and T_a in the Roberts and Harrison (1999) study may be attributable to the overall higher and broader range of T_{th} values encountered, with the lowest value at 38°C (Fig. 11). In the Heinrich (1980b) study, mean T_{th} fell either above or below the 36 to 41.5°C range.

Metabolic rates in our midsummer experiment (agitation effects) are similar to those for honeybees measured in midsummer in Arizona (Harrison et al., 1996a,b; fig. 9 in Roberts and Harrison, 1999), while values for our late-season measurements (primary dataset) are intermediate between

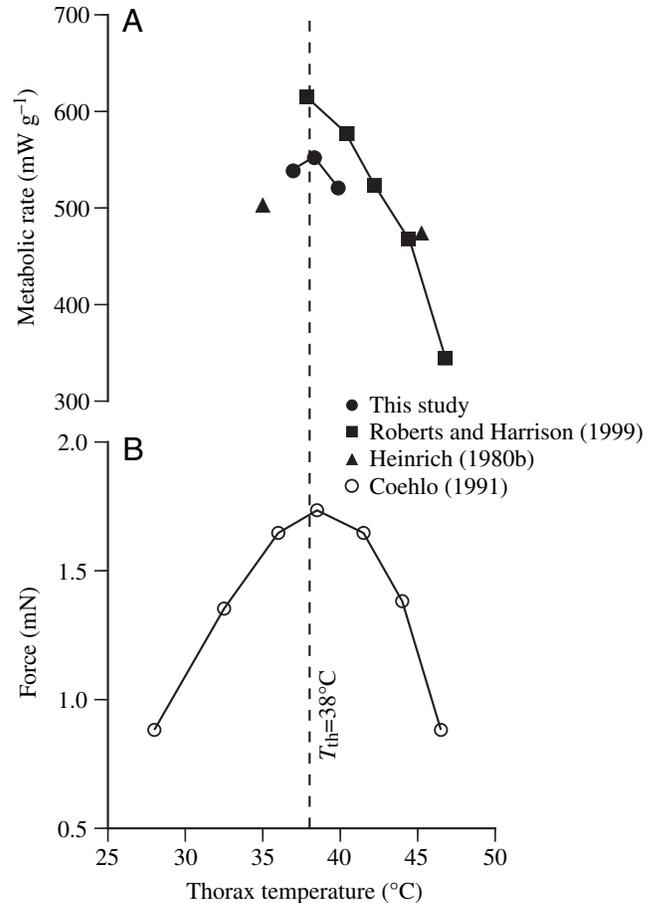


Fig. 11. Comparison of the relationships of force production and flight metabolic rate with thorax temperature for honeybees. (A) The relationship between flight metabolic rate and thorax temperature for voluntary flight from three studies. Data from Roberts and Harrison (1999) are re-analyzed by discrete temperature ranges. (B) The relationship between force production and thorax temperature in tethered flight (from Coelho, 1991).

those collected in midsummer and in temperate midwinter (Harrison et al., 2001; Harrison and Fewell, 2002). The 15% difference between our datasets is several times greater than has been associated with colony or genotype effects (Harrison et al., 1996a; Harrison and Fewell, 2002), but leaves open the possibilities of effects of season (Harrison et al., 2001) or foraging task (Feuerbacher et al., 2003).

Dependence of body temperature on air temperature

The slope of T_{th} on T_a between 18 and 38°C was 0.18, or shallower by at least half those found for honeybees in untethered flight; previous values for indoor studies range from 0.39 to 0.52 (Heinrich, 1979, 1980b; Harrison et al., 1996; Roberts and Harrison, 1999), and for outdoor studies from 0.37 to 0.44 (Cooper et al., 1985; Coelho, 1991; Feuerbacher et al., 2003). What could explain the twofold difference in the slope of T_{th} on T_a between our study and others? A direct approach to this question would be to calculate heat budgets for different T_a values. We do not follow that approach because of the lack

Table 1. Comparison of the change in mean metabolic energy production per wingbeat with air temperature for three insect species

Study	Species	T_a range (°C)	Decline in energy production (%)	
			Total	Per °C
This study	<i>A. mellifera</i>	20–37	14.4	0.85
Harrison et al., 1996	<i>A. mellifera</i>	21–38	35	1.9
Roberts and Harrison, 1998	<i>Centris pallida</i> (Hymenoptera: Anthophoridae)	27–34.5	25	3.3
May, 1995	<i>Anax junius</i> (Odonata: Aeshnidae)	21–26.5	16	3.0

T_a , air temperature.
Energy production (J wingbeat^{-1}) was measured over the full T_a range.

of temporal synchrony of the body temperature measurements with FMR and evaporative heat loss (EHL) data, critical for animals that exhibit rapid heating and cooling rates, and because we lack data for surface temperature and convective heat loss. However, it is useful to compare our results with previous research in terms of what we do know about all possible paths of heat exchange. Sources of heat gain in our case include metabolic heat production and short-wave radiation. These gains must be balanced by heat losses that include net long-wave radiation, convection and evaporation. In the only other study to measure FMR, EHL and body segment temperatures of honeybees in voluntary flight (Roberts and Harrison, 1999), mean T_{th} at $T_a=21^\circ\text{C}$ was only 0.6°C higher than in our study despite mean FMR values that were about 100 mW g^{-1} (16%) higher, EHL that was somewhat (about 30 mW g^{-1}) lower, and long-wave heat flux that was presumably similar. This roughly 70 mW g^{-1} , or net remaining difference, may be attributable to the calculated short-wave heat gain value range of $58\text{--}84\text{ mW g}^{-1}$ in our study. At higher T_a values ($34\text{--}39^\circ\text{C}$), bees in our study defended lower T_{th} values; at $T_a=38^\circ\text{C}$, predicted T_{th} was 40.2°C in our study and 44.4°C in Roberts and Harrison (1999). This difference in T_{th} corresponds to differences between the two studies in the responses of temperature excess ratios and EHL to T_a . In our case, R_h and R_{ab} became lower and more variable at T_a values above 34°C (Fig. 6), while in the Roberts and Harrison (1999) study this change did not occur until T_a exceeded 39°C ; EHL reached $100\text{--}200\text{ mW g}^{-1}$ at T_a values of $35\text{ to }36^\circ\text{C}$ in our case but only at $30\text{--}40^\circ\text{C}$ in Roberts and Harrison (1999). Curiously, EHL also increased as T_a values fell below 21°C ; it is possible that this was due to excretion of excess water resulting from a positive water balance (see Nicolson and Louw, 1982; Bertsch, 1984) and contributed little to EHL.

The head serves as a thermal window for excess thoracic heat through evaporaton of regurgitated nectar (Esch, 1976; Heinrich, 1980a,b). At air temperatures below 30°C , mean T_h excess was about 7°C . However, at T_a values from $35\text{ to }38^\circ\text{C}$, mean T_h was more than 2°C lower than T_a . This crossover occurs at a T_a value $>6^\circ\text{C}$ lower than in indoor measurements of untethered honeybees (Heinrich, 1980b; Roberts and Harrison, 1999).

T_{ab} closely tracked T_a when the latter was $<30^\circ\text{C}$, but

showed a surprising decline to about 4°C below T_a when the latter was between $35\text{ and }38^\circ\text{C}$. There is no previous evidence that the abdomen plays more than a minor role as a heat exchanger in honeybees (Heinrich, 1980b). Honeybees maintain impressively high T_{th} despite small body size and lack of insulation, at least partly by preventing the loss of heat to the abdomen, which is joined to the thorax by a narrow petiole that has a countercurrent heat exchanger (see Heinrich, 1980b); there is no evidence that this heat exchange is circumvented at higher air temperatures in order to take advantage of the abdomen as a thermal window, as occurs in bumblebees (Heinrich, 1976, 1980b). Nevertheless, T_{ab} did drop below T_a when the latter was $>42^\circ\text{C}$ in previous indoor measurements (Heinrich, 1980b; Roberts and Harrison, 1999), and is associated with higher total EHL, for which several possible mechanisms have been proposed (Roberts and Harrison, 1999).

Regulation of lower thoracic temperatures

Why did honeybees in our study regulate lower and more stable T_{th} at higher air temperatures, apparently through higher EHL? Honeybees flying at air temperatures where they could maintain T_{th} at or near the 38°C level, associated with maximum metabolic rate and force production, do not always do so. Attacking bees regulate T_{th} at about 38°C , whereas the mean T_{th} of outgoing foragers from the same hive is about 3°C lower (Heinrich, 1979), and bees imbibing $40\text{--}60\%$ sucrose maintain T_{th} at 36°C , while bees imbibing $10\text{--}30\%$ sucrose maintain T_{th} of only 33°C (Waddington, 1990). Those studies were made at lower air temperatures, where allowing T_{th} to fall results in a lower rate of energy use. At higher air temperatures, however, similar energy savings could be realized by limiting active evaporative heat loss and allowing T_{th} to rise above 38°C , yielding dividends of improved water balance and greater flight range (see Cooper et al., 1985). Direct experimental evidence for such a strategy is lacking, but the observation that T_{th} decreased as wing loading increased for honeybees foraging at T_a values between $35\text{ and }40^\circ\text{C}$ (Cooper et al., 1985) is suggestive. Since flying honeybees can have FMR values as high as 800 mW g^{-1} , but can maintain flight with FMR as low as about 300 mW g^{-1} (Coelho, 1991; Harrison and Fewell, 2002), there appears to be a considerable margin within which a trade-off between energy and water

conservation on the one hand and mechanical power production on the other could be varied through the active mechanism of evaporative heat loss at high T_a .

Dependence of wingbeat frequency on air temperature

In studies that have measured WBF and FMR during hovering, agitated or tethered flight, the two variables have similar associations with T_a in honeybees (Esch, 1976; Feller and Nachtigall, 1989; Harrison et al., 1996; Roberts and Harrison, 1999) and in other insects (May, 1995; Roberts et al., 1998; Borrel and Medeiros, 2004). However, as with FMR, the slope of the relationship between WBF and T_a for honeybees is variously found to be strongly positive (Feller and Nachtigall, 1989; Esch, 1976), slightly positive (this study), zero (Spangler, 1992) or negative (Spangler, 1992; Harrison et al., 1996; Roberts and Harrison, 1999). These differences appear to be explained by differences in T_{th} range between studies. Where positive slopes of WBF on T_a are reported, T_{th} values all fell below 36°C (Esch, 1976; Feller and Nachtigall, 1989), and where negative slopes are reported, T_{th} values were mostly above 37°C; in our study, where the slope was only slightly positive, T_{th} fell between 36 and 40°C. Thus, the relationship of WBF to T_{th} appears to follow the same general pattern as the relationships of FFP and FMR to T_{th} .

Dependence of energy expenditure per wingbeat on air temperature

This is the first report of a temperature effect on energy expenditure per wingbeat for honeybees in voluntary flight; as air temperature increased from 20 to 37°C for first-quality flight, energy expenditure per wingbeat diminished by 14.4%. An examination of data from other studies, however, suggests that this phenomenon may not be uncommon among flying insects (Table 1). In the case of honeybees, the finding may explain an apparent discrepancy noted between tethered flight experiments (Heinrich, 1993): although the oxygen consumption per action potential is independent of T_a in both honeybees (Bastian and Esch, 1970) and bumblebees (Kammer and Heinrich, 1974), mechanical power output at a given action-potential frequency increases with temperature (Esch et al., 1975). This is consistent with the positive relationship of T_a and muscle efficiency in hovering orchid bees (Borrell and Medeiros, 2004). Since honeybees are myogenic fliers, with stretch-activated wing muscles, this effect is probably not due to greater overlap of muscle contractions at lower temperatures, leaving open the possibility that muscle viscosity or internal friction decreases at higher temperatures, or that elastic energy storage is affected by temperature (Heinrich, 1993; Borrell and Medeiros, 2004).

Could agitation be a confounding factor?

For the full sample of our primary data set, honeybees spent an average of 52% of the measurement period in flight, with this value declining at higher T_a values (Fig. 8), at which foragers will often cease flight in order to cool (Cooper et al., 1985). As the fraction of time spent in flight decreased, the

slope of FMR on T_a became steeper (Fig. 8). For bees flying less than 40% of the time, the slope fell between that for workers that were not flying at all (Cahill and Lustick, 1976) and that for bees that spent more than 80% of the time flying (Fig. 8). Workers that are not flying nevertheless maintain high thorax temperatures by shivering their wing muscles (Cahill and Lustick, 1976); at lower air temperatures, this behavior has a metabolic cost similar to that of flight (Figs 8, 9), but as air temperature increases, shivering diminishes and non-flight metabolic rate declines to a small fraction of its value at lower T_a (Fig. 8; Cahill and Lustick, 1976). Thus, to the extent that non-flight behavior is included in respirometry measurements of honeybee workers, we might expect a more negative slope of metabolic rate on T_a , and that this effect would be distinct from that of T_{th} on FMR (Fig. 11). This may explain why, for bees with T_{th} in the 36–41.5°C range over which FMR and FFP are relatively unchanged, there is an inverse relationship between FMR and T_a for bees under constant agitation (for $T_a=20\text{--}27^\circ\text{C}$, $\text{W g}^{-1}\text{thorax}=2.53-0.048T_a$, $N=69$, $r^2=0.23$, $P<0.0001$; data from Harrison et al., 1996) but not for bees in voluntary flight [for $T_a=20\text{--}29^\circ\text{C}$, $\text{mW g}^{-1}=674.1-3.19T_a$, $N=10$, $r^2=0.03$, $P=0.64$; data from Roberts and Harrison (1999); see Fig. 2A, all measurements, in the present study]. This is supported by the results of our separate agitation effects experiment, in which bees were agitated only as much as necessary to keep them in the air, with bees' willingness to fly without agitation as our primary independent variable. The slope of FMR on T_a for bees in our study that required constant or frequent agitation to remain airborne is similar to that for bees that were constantly agitated regardless of willingness to fly (Harrison et al., 1996), suggesting that metabolic measurements made under constant agitation, while valuable where a large sample size is required under constant conditions, may not be representative of flight where T_a is an independent variable.

Indoor vs outdoor flight

In outdoor measurements, the incidence of voluntary flight was about twice that for indoor measurements. Foraging honeybees are known to navigate using familiar landmarks and polarized light (Dyer, 1996; Esch and Burns, 1996; Wehner et al., 1997; Srinivasan et al., 1996, 1997), cues that were present in our study but not in other respirometry measurements of honeybees. Honeybees also have a high flicker fusion frequency (Miall, 1978), and show higher WBF and slower, more interrupted flight under fluorescent lighting of 100 Hz, similar to that in many laboratories, than under fluorescent lighting of 300 Hz (van Praagh, 1972). In addition, honeybees judge distance to objects during flight by motion parallax (Srinivasan et al., 1991, 1996), and in our outdoor measurements bees had a largely unobstructed view of natural objects at natural distances. When, during the agitation effects experiment, we briefly placed visual barriers close to the side of the chamber the bee was facing, the animal nearly always re-oriented itself to face in a direction with an unobstructed view. It was probable, though, that bees could also perceive

the chamber wall, possibly because of the ripples in the glass surface, since most sustained circling, bobbing or hovering flight toward the center of the chamber rather than persistently flying against the glass.

The salient difference in methods between our study and others of honeybees in voluntary flight under confinement was the presence of more nearly natural visual stimuli, and the principal difference in results was the bees' defense of a narrower T_{th} range which, in turn, appears to explain our finding of independence of FMR and T_a . We cannot conclude that any of our findings other than the frequency of voluntary flight were affected by our outdoor location. However, providing a more natural visual environment may be more important than previously recognized for obtaining representative flight under containment, and partitioning the effects of different components of the full suite of natural visual stimuli, may help explain remaining differences in results between respirometry studies of honeybees, as well as other insects, in flight.

List of symbols and abbreviations

EHL	evaporative heat loss
FMR	flight metabolic rate
FTF	fraction of the measurement period spent in flight
MR	metabolic rate
R	temperature excess ratio
T_a	air temperature
T_{ab}	abdomen temperature
T_h	head temperature
T_{th}	thorax temperature
WBF	wingbeat frequency

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