

## Oxygen consumption rates in hovering hummingbirds reflect substrate-dependent differences in P/O ratios: carbohydrate as a 'premium fuel'

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### Summary

The stoichiometric relationship of ATP production to oxygen consumption, i.e. the P/O ratio, varies depending on the nature of the metabolic substrate used. The latest estimates reveal a P/O ratio approximately 15% higher when glucose is oxidized compared with fatty acid oxidation. Because the energy required to produce aerodynamic lift for hovering is independent of the metabolic fuel oxidized, we hypothesized that the rate of oxygen consumption,  $\dot{V}_{O_2}$ , should decline as the respiratory quotient, RQ ( $\dot{V}_{CO_2}/\dot{V}_{O_2}$ ), increases from 0.71 to 1.0 as hummingbirds transition from a fasted to a fed state. Here, we show that hovering  $\dot{V}_{O_2}$  values in rufous (*Selasphorus rufus*) and Anna's hummingbirds (*Calypte anna*) are significantly greater when fats are metabolized (RQ=0.71) than when carbohydrates are used (RQ=1.0). Because hummingbirds gained mass during our experiments, making mass a confounding variable, we

estimated  $\dot{V}_{O_2}$  per unit mechanical power output. Expressed in this way, the difference in  $\dot{V}_{O_2}$  when hummingbirds display an RQ=0.71 (fasted) and an RQ=1.0 (fed) is between 16 and 18%, depending on whether zero or perfect elastic energy storage is assumed. These values closely match theoretical expectations, indicating that a combination of mechanical power estimates and 'indirect calorimetry', i.e. the measurement of rates of gas exchange, enables precise estimates of ATP turnover and metabolic flux rates *in vivo*. The requirement for less oxygen when oxidizing carbohydrate suggests that carbohydrate oxidation may facilitate hovering flight in hummingbirds at high altitude.

Key words: P/O ratio, carbohydrate, fatty acid, hummingbird, oxygen consumption.

### Introduction

In animals engaged in high-intensity, aerobic exercise, >90% of O<sub>2</sub> consumption rates (i.e.  $\dot{V}_{O_2}$ ) are accounted for by mitochondrial respiration in locomotory muscles (Suarez, 1992; Taylor, 1987). For decades, it has been recognized by physiologists that conversion of such  $\dot{V}_{O_2}$  values to the equivalent values of energy expenditure in watts or joules requires consideration of the metabolic substrate(s) used (e.g. Kleiber, 1961; Schmidt-Nielsen, 1990). On the other hand, biochemists have empirically determined mitochondrial P/O ratios (moles ATP phosphorylated per mole oxygen atom consumed) and found that these depend upon the nature of the substrates oxidized. The latest estimates, based on a combination of empirical data and theoretical considerations, reveal an ~15% higher cellular P/O ratio when glucose is oxidized compared with fatty acid oxidation (Brand, 1994; Brand, 2005). This substrate-dependent difference in the efficiency of aerobic ATP production has been observed in isolated, perfused hearts that, when provided with fatty acids to oxidize, elevate their  $\dot{V}_{O_2}$  above rates obtained when oxidizing glucose (Korvald et al., 2000; Neely and Morgan,

1974). Such differences in P/O ratios have also been observed *in vitro* using mitochondria isolated from hummingbird flight muscles (Suarez et al., 1986).

At one or more stages in their education, biologists are often required to memorize P/O ratios and other stoichiometric relationships in biochemistry. Apart from turning a fascinating subject into a dry exercise in memorization, the relevance of stoichiometric relationships to the lives of real animals is often presented as a matter of faith, i.e. a matter that cannot be subjected to empirical test, except in cell-free extracts. So, an important question that we address here is whether substrate-dependent P/O ratios influence whole animal performance and whether they have any relevance to behavioral ecology.

Hummingbirds offer a unique opportunity to determine whether the substrate dependence of P/O ratios is manifested in whole animals and what the significance of this might be. Their flight muscles consist exclusively of fast-twitch, oxidative fibers (Grinyer and George, 1969; Suarez et al., 1991; Suarez, 1992). Thus, >90% of their  $\dot{V}_{O_2}$  during flight is accounted for by mitochondrial respiration in a single cell type. In addition, it is possible to alter the relative contributions of

carbohydrate and fat to the fueling of mitochondrial respiration through manipulation of their dietary status. Fasted hummingbirds rely heavily on fatty acid oxidation and rapidly switch to carbohydrate oxidation when dietary sucrose becomes available, oxidizing newly ingested sucrose almost exclusively to fuel foraging flight (Suarez et al., 1990; Welch et al., 2006). All else being equal, the mechanical power requirements for hovering flight should remain constant irrespective of the fuel oxidized. In biochemical terms, the ATP turnover (i.e. synthesis = hydrolysis) rate required for a unit mass of bird to hold itself aloft should be independent of the nature of the fuel oxidized. Assuming a negligible contribution of protein to the support of metabolism (Vaillancourt et al., 2005), RQ ( $=\dot{V}_{CO_2}/\dot{V}_{O_2}$ ) may be used to infer the relative contributions of carbohydrate and fat to the support of metabolism, with an RQ of 0.71 indicating exclusively fat oxidation and an RQ of 1.0 indicating exclusively carbohydrate oxidation. The difference in P/O ratio between carbohydrate and fat oxidation leads to the hypothesis that  $\dot{V}_{O_2}$  should decline by 15% as the RQ increases from 0.71 to 1.0.

Recently reported mismatches between heat production rates predicted from indirect calorimetry, determined through measurement of rates of whole-animal gas exchange, and those measured by 'direct calorimetry' have raised doubt concerning the validity of indirect calorimetry as a means by which to estimate metabolic rate (Walsberg and Hoffman, 2005). However, rates of metabolic heat production are not predictive of rates of ATP turnover, nor are they useful for estimating flux rates through the pathways of substrate oxidation. Here, we consider the results of indirect calorimetry in terms of the biochemical meaning of the term 'metabolic rate'. We demonstrate how this, in combination with estimates of mechanical power output, leads to novel insights concerning the energetics of hummingbird hovering flight.

### Materials and methods

The study was conducted with rufous hummingbirds (*Selasphorus rufus* Gmelin 1788; body mass  $M_b=4.1\pm 0.3$  g;  $N=6$ , four male/two female) captured in Inyo, Mono and Santa Barbara Counties in California, USA and Anna's hummingbirds (*Calypte anna* Lesson 1829;  $M_b=4.4\pm 0.6$  g;  $N=4$ , two male/two female) captured in Santa Barbara County, California, USA. All hummingbirds were captured using a modified Hall trap (Russell and Russell, 2001) and housed at the UCSB Aviary in individual outdoor, wire-mesh enclosures measuring 1.8 m tall  $\times$  0.6 m wide  $\times$  2.4 m long. Birds were fed *ad libitum* on a 13% (w/v) solution of Nektar-Plus (Guenter Enderle, Tarpon Springs, FL, USA) supplemented with beet sugar (5% w/v). Birds were subjected to natural photophase and ambient weather conditions. Capture, housing and experimental protocols were approved by the University of California, Santa Barbara Institutional Animal Care and Use Committee (Protocol 672).

Experiments were performed in an enclosure measuring 0.92 m wide  $\times$  0.54 m high  $\times$  0.51 m deep, in the laboratory

at a mean temperature of  $23.9\pm 0.4^\circ\text{C}$ . Data collection took place between April and August 2006 between 06.00 h and 11.00 h. Prior to the experiment, each hummingbird was fasted overnight to ensure that it would be oxidizing primarily fat at the beginning of the hour-long period of data collection (Suarez et al., 1990; Welch et al., 2006) as well as to ensure that it would be motivated to feed. Hummingbirds were active and sporadically hovering for a period of at least 20 min prior to the beginning of data collection. Experiments were repeated using each individual approximately 1–2 weeks later, with the exception of the two male Anna's hummingbirds (*C. anna*). Hummingbirds were offered the Nektar-Plus and beet sugar solution for a period of one hour. Oxygen consumption ( $\dot{V}_{O_2}$ ) and carbon dioxide production ( $\dot{V}_{CO_2}$ ) rates were determined by open-flow respirometry during the first hour of access to feeder solution following the overnight fast. The respirometric system used follows one described elsewhere (Bartholomew and Lighton, 1986; Suarez et al., 1990; Welch et al., 2006). Briefly, we constructed the feeder such that birds had to hover and insert their heads into a plastic tube (that functioned as a mask) to gain access to the end of a 20 ml disposable syringe, filled with feeder solution. Plastic tubing was attached to the side of the mask, allowing incurrent air to be drawn and then passed through a column of Drierite<sup>TM</sup> (W. A. Hammond Drierite, Xenia, OH, USA) to scrub water vapor before entering the carbon dioxide analyzer (CA-2A; Sable Systems International, Las Vegas, NV, USA). After leaving the carbon dioxide analyzer, air passed through a Drierite<sup>TM</sup>-Ascarite<sup>TM</sup>-Drierite<sup>TM</sup> column (Ascarite II; Arthur H. Thomas, Philadelphia, PA, USA), to scrub any carbon dioxide and additional water from the line, and then into the oxygen analyzer (FOXBOX; Sable Systems International). Air flow was maintained by a mechanism internal to the FOXBOX (thus, after the removal of water vapor) at a rate of  $1200\text{ ml min}^{-1}$ . The presence of the hummingbird's head inside the mask was detected by interruption of an infrared beam crossing the front edge of the mask. The only available perch in the cage was placed on top of a balance, allowing recording of bird mass. Mass was estimated for each feeding event as the average of mass values immediately prior to and following that feeding event. Oxygen analyzer, carbon dioxide analyzer, flow rate, infrared beam and balance analog output were converted into a digital signal (Universal Interface II; Sable Systems International), which was then fed to a computer. Data were recorded at 0.05-s intervals for 1 h using Expedata software (v. 1.0.17; Sable Systems International).

Immediately before data collection, the oxygen analyzer was calibrated with well-mixed ambient air drawn through the mask in the absence of a hummingbird. The carbon dioxide analyzer was calibrated with  $\text{CO}_2$ -free nitrogen gas (zero gas) and 0.5%  $\text{CO}_2$  in nitrogen gas (Praxair, Danbury, CT, USA). In each case, tubing was removed directly downstream of the mask and held inside a small reservoir into which flowed the calibration gas at a rate in excess of the flow rate of air pulled through the respirometry system.

Rates of oxygen consumption ( $\dot{V}_{O_2}$ ) and carbon dioxide

production ( $\dot{V}_{CO_2}$ ) were determined from raw oxygen and carbon dioxide traces as described previously (Suarez et al., 1990; Welch et al., 2006). STP-corrected oxygen depletion and carbon dioxide enrichment associated with each feeding event were determined by first correcting the traces by subtracting baseline values (determined as the linear extrapolation of points directly before and after the feeding event in question). These baseline-corrected data were then converted to ml of gas by application of standard equations (Withers, 1977). Rates of gas consumption or production were determined by dividing the volume of gases respired by the time the hummingbird's head was inside the mask (estimated as the period of time that the infrared beam was occluded). All feeding events that lasted for less than 1 s in duration were excluded from analysis. All means are reported  $\pm$  s.d.

### Results

During each hour of data collection, birds fed between six and 20 times (mean=10.8 $\pm$ 3.4;  $N=18$ ). Fig. 1 shows that mass-specific oxygen consumption rate ( $\dot{V}_{O_2}/M_b$ ) varies significantly (1-way ANOVA analysis; data not shown) in relation to RQ. However, it is important to note that each bird gained mass during the hour (*C. anna*, 0.33 $\pm$ 0.15 g, range=0.16–0.57 g,  $N=6$ ; *S. rufus*, 0.28 $\pm$ 0.11 g, range=0.16–0.47 g,  $N=12$ ).

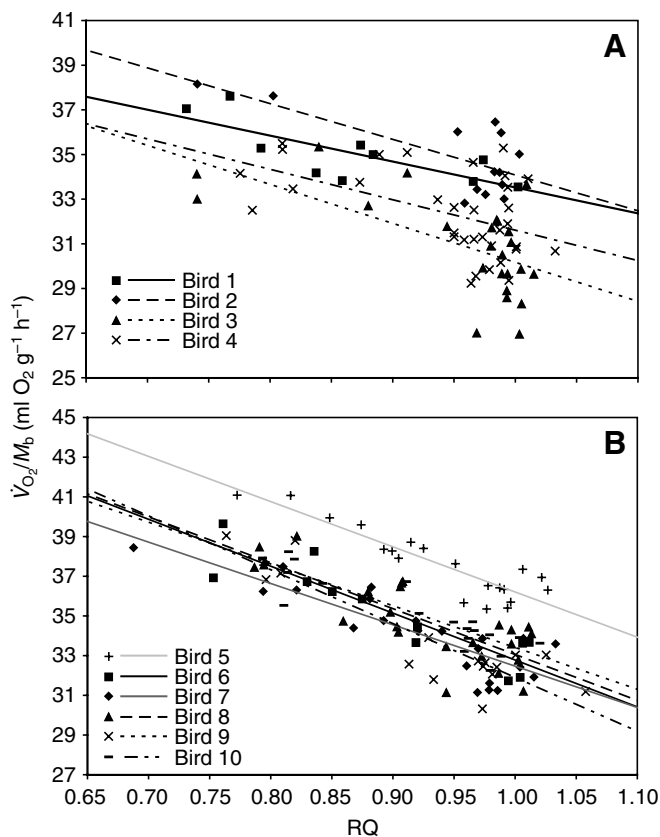


Fig. 1. Mass-specific oxygen consumption rate ( $\dot{V}_{O_2}/M_b$ ) in relation to respiratory quotient (RQ) in (A) Anna's (*C. anna*) and (B) rufous hummingbirds (*S. rufus*).

Aerodynamic theory predicts that mass-specific power output requirements for hovering should vary as a complex function of the mass being lifted (Ellington, 1984a; Ellington, 1984b; Ellington, 1984c; Ellington, 1984d; Ellington, 1984e; Ellington, 1984f). Total hovering power requirements are a function of the individual component power requirements, including the energy required to overcome profile drag forces on the wings [profile power ( $P_{pro}$ )], the energy required to move sufficient air in order to overcome the downward pull of gravity [induced power ( $P_{ind}$ )] and the energy necessary to accelerate the wings during the first half of a half-stroke [inertial power ( $P_{acc}$ )]. If the kinetic energy of the wings during the first half of each half-stroke can be completely stored as elastic strain energy during the second half of the half-stroke and then released at the beginning of the following stroke (perfect elastic storage), then  $P_{acc}$  will be zero and total hovering power requirement ( $P_{per}$ ) will equal  $P_{pro}+P_{ind}$ . Instead, if none of the kinetic energy of the wings is recovered for subsequent strokes (zero elastic storage), then total hovering power requirement ( $P_{zero}$ ) will equal  $(P_{pro}+P_{ind}+P_{acc})/2$ . Actual hovering power requirements are not empirically known for hovering hummingbirds but are likely to be somewhere between estimated  $P_{per}$  and  $P_{zero}$  values. We estimated  $P_{per}$  and  $P_{zero}$  using equations in Ellington (Ellington, 1984f). Body mass was measured for each bird during each feeding event (as described above) whereas other morphological and kinematic parameters required for power estimates were taken from average species- and gender-specific values of rufous (Altshuler, 2006; Altshuler and Dudley, 2003) and Anna's hummingbirds (D.L.A., unpublished data).

We controlled for the chain effects of  $M_b$  on muscle power requirements and therefore on  $\dot{V}_{O_2}$  by dividing mass-specific  $\dot{V}_{O_2}$  ( $\dot{V}_{O_2}/M_b$ ) by mass-specific mechanical power requirements for hovering assuming perfect elastic energy storage ( $P_{per}$ ). This correction, oxygen consumption rate per unit hovering power output assuming perfect elastic energy storage in ml  $O_2$   $h^{-1}$   $W^{-1}$ , is summarized as follows:

$$\dot{V}_{O_2} / W_{per} = (\dot{V}_{O_2} / M_b) / P_{per}, \quad (1)$$

where  $\dot{V}_{O_2}/M_b$  is in ml  $O_2$   $g^{-1}$   $h^{-1}$  and  $P_{per}$  is in  $W$   $g^{-1}$ . To obtain oxygen consumption rate per unit hovering power output assuming zero elastic energy storage in ml  $O_2$   $h^{-1}$   $W^{-1}$ , we simply substituted  $P_{zero}$  for  $P_{per}$ , as follows:

$$\dot{V}_{O_2} / W_{zero} = (\dot{V}_{O_2} / M_b) / P_{zero}, \quad (2)$$

where  $\dot{V}_{O_2}/M_b$  is in ml  $O_2$   $g^{-1}$   $h^{-1}$  and  $P_{zero}$  is in  $W$   $g^{-1}$ . The effect of variations in mechanical power output assuming either perfect or zero elastic energy storage on carbon dioxide production rate can similarly be removed by using mass-specific carbon dioxide production rate ( $\dot{V}_{CO_2}/M_b$ ) in place of  $\dot{V}_{O_2}/M_b$  in Eqns 1 and 2 to solve for carbon dioxide production rate per unit hovering power output ( $\dot{V}_{CO_2}/W_{per}$  and  $\dot{V}_{CO_2}/W_{zero}$ ).

There was no significant effect of trial day on  $\dot{V}_{O_2}/W_{per}$  and  $\dot{V}_{O_2}/W_{zero}$  or  $\dot{V}_{CO_2}/W_{per}$  and  $\dot{V}_{CO_2}/W_{zero}$  in either Anna's ( $\dot{V}_{O_2}/W_{per}$ :  $F_{1,3}=0.0081$ ,  $P=0.9287$ , ANOVA;  $\dot{V}_{O_2}/W_{zero}$ :

$F_{1,3}=0.0082$ ,  $P=0.9281$ , ANOVA;  $\dot{V}_{CO_2}/W_{per}$ :  $F_{1,3}=0.0058$ ,  $P=0.9395$ , ANOVA;  $\dot{V}_{CO_2}/W_{zero}$ :  $F_{1,3}=0.0028$ ,  $P=0.9576$ , ANOVA) or rufous hummingbirds ( $\dot{V}_{O_2}/W_{per}$ :  $F_{5,5}=0.4030$ ,  $P=0.8456$ , ANOVA;  $\dot{V}_{O_2}/W_{zero}$ :  $F_{5,5}=0.4024$ ,  $P=0.8460$ , ANOVA;  $\dot{V}_{CO_2}/W_{per}$ :  $F_{5,5}=0.2425$ ,  $P=0.9425$ , ANOVA;  $\dot{V}_{CO_2}/W_{zero}$ :  $F_{5,5}=0.2439$ ,  $P=0.9418$ , ANOVA). The data for each individual from the two trial days were therefore combined for further analysis. Table 1 shows results of a linear model of the effect of RQ on oxygen consumption rate per unit hovering power output and carbon dioxide production rate per unit hovering power output for both Anna's and rufous hummingbirds. For Anna's hummingbirds, with individual hummingbird as a random effect within a linear model, the effect of metabolic substrate, as indicated by RQ, on  $\dot{V}_{O_2}/W$  and  $\dot{V}_{CO_2}/W$  was highly significant ( $P<0.0001$ ) when either perfect or zero elastic storage was assumed (Table 1). There was no difference in the relationship of either  $\dot{V}_{O_2}/W$  or  $\dot{V}_{CO_2}/W$  to RQ between Anna's hummingbirds (RQ  $\times$  individual interaction term;  $P>0.9052$ ; see Table 1), indicating that the nature of the effect of metabolic substrate on oxygen consumption or carbon dioxide production rate was constant across individuals. Results were similar for rufous hummingbirds (Table 1). With individual hummingbird as a random effect within a linear model, the effect of metabolic substrate, as indicated by the RQ, on  $\dot{V}_{O_2}/W$  and  $\dot{V}_{CO_2}/W$  was also highly significant ( $P<0.0001$ ) assuming either perfect or zero elastic storage (Table 1). As with Anna's hummingbirds, there was no difference in the relationship of either  $\dot{V}_{O_2}/W$  or  $\dot{V}_{CO_2}/W$  to RQ between rufous hummingbirds (RQ  $\times$

individual interaction term;  $P>0.1063$ ; see Table 1), indicating that the nature of the effect of metabolic substrate on oxygen consumption or carbon dioxide production rate was also constant across individuals within this taxon.

Linear functions describing the relationship of  $\dot{V}_{O_2}/W_{per}$  or  $\dot{V}_{O_2}/W_{zero}$  to RQ for each hummingbird (Figs 2, 3) can be used to determine the mass-corrected oxygen consumption rate when burning solely fat (RQ=0.71) or carbohydrate (RQ=1.0). The relative difference between these values can then be compared to the relative difference in P/O ratios (Brand, 2005) under these two conditions. Table 2 shows the predicted  $\dot{V}_{O_2}/W_{per}$  and  $\dot{V}_{O_2}/W_{zero}$  values as a function of metabolic substrate for each individual. For Anna's hummingbirds, percentage differences between the predicted  $\dot{V}_{O_2}/W$  values at an RQ of 0.71 and 1.0 averaged  $18.0\pm 0.6\%$  ( $N=4$ ) when perfect elastic storage ( $P_{per}$ ) was assumed and  $16.4\pm 0.8\%$  ( $N=4$ ) when zero elastic storage ( $P_{zero}$ ) was assumed. For rufous hummingbirds, percent differences between the predicted  $\dot{V}_{O_2}/W$  values at an RQ of 0.71 and 1.0 averaged  $16.8\pm 1.7\%$  ( $N=6$ ) when  $P_{per}$  was assumed and  $16.2\pm 2.1\%$  ( $N=6$ ) when  $P_{zero}$  was assumed.

### Discussion

In the present study, we show that substrate-dependent variation in the P/O ratio, resulting from differences in the stoichiometry of biochemical reactions involved in substrate oxidation, can be detected using indirect calorimetry. The experimental methodology employed here cannot be used to

Table 1. Linear regression model of the effects of respiratory quotient (RQ) and individual on  $O_2$  consumption rate per unit hovering power output ( $\dot{V}_{O_2}/W$ )

| Effect                 | O <sub>2</sub> consumption rate<br>per unit hovering work |          | CO <sub>2</sub> production rate<br>per unit hovering work |          |
|------------------------|---|----------|---|----------|
|                        | F ratio (d.f.)  | P value  | F ratio (d.f.)  | P value  |
| <i>C. anna</i>         |   |          |   |          |
| Using $P_{per}$        |   |          |   |          |
| Individual             | Random effect   |          | Random effect   |          |
| RQ                     | 76.4853 (1,1)   | <0.0001* | 25.4954 (1,1)   | <0.0001* |
| RQ $\times$ individual | 0.1522 (3,3)  | 0.9280   | 0.1366 (3,3)  | 0.9379   |
| Using $P_{zero}$       |   |          |   |          |
| Individual             | Random effect   |          | Random effect   |          |
| RQ                     | 53.4544 (1,1)   | <0.0001* | 30.4297 (1,1)   | <0.0001* |
| RQ $\times$ individual | 0.1867 (3,3)  | 0.9052   | 0.0515 (3,3)  | 0.9844   |
| <i>S. rufus</i>        |   |          |   |          |
| Using $P_{per}$        |   |          |   |          |
| Individual             | Random effect   |          | Random effect   |          |
| RQ                     | 308.5417 (1,1)  | <0.0001* | 152.3897 (1,1)  | <0.0001* |
| RQ $\times$ individual | 0.4684 (5,5)  | 0.7990   | 0.8650 (5,5)  | 0.5074   |
| Using $P_{zero}$       |   |          |   |          |
| Individual             | Random effect   |          | Random effect   |          |
| RQ                     | 286.4036 (1,1)  | <0.0001* | 170.9568 (1,1)  | <0.0001* |
| RQ $\times$ individual | 1.3585 (5,5)  | 0.2462   | 1.8688 (5,5)  | 0.1063   |

Significant results are marked by an asterisk.

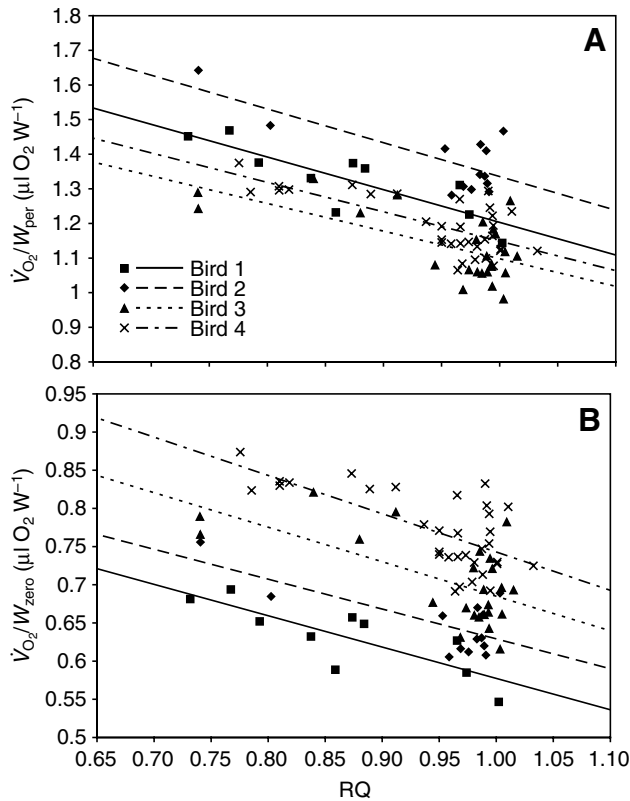


Fig. 2. Oxygen consumption rate per unit hovering power output ( $\dot{V}_{O_2}/W$ ) in relation to respiratory quotient (RQ) in Anna's hummingbirds (*C. anna*) assuming (A) perfect ( $P_{per}$ ) or (B) zero ( $P_{zero}$ ) elastic storage.

measure substrate-dependent variation in P/O ratios *per se* but, instead, reveals consistency between the results of *in vivo* and *in vitro* studies. The difference in oxygen consumption rate as a function of the metabolic substrate oxidized *in vivo* is similar to that predicted from the P/O ratios obtained using isolated hummingbird flight muscle mitochondria (Suarez et al., 1986) as well as the latest consensus values calculated for whole cells (Brand, 2005).

Detection of substrate-dependent variation in the P/O ratio is feasible in hovering hummingbirds in large part because they so rapidly shift from displaying an RQ near 0.71, indicating primarily fat oxidation, to an RQ near 1.0, indicating primarily carbohydrate oxidation. Hummingbirds store excess energy primarily by converting ingested sugars into fat (Carpenter et al., 1993; Odum et al., 1961). As Frayn details (Frayn, 1983), the RQ associated with carbon fixation and the oxidation of fuels to provide the ATP necessary to convert sugars into lipids is approximately 5.6. Thus, if lipogenesis is activated shortly after sugar ingestion, it is possible that the RQ that a hummingbird displays during repeated hover-feeding may be elevated by both carbohydrate oxidation (to fuel hovering flight) and the metabolic processes associated with lipogenesis. However, the effect of lipogenesis on hovering hummingbird RQ is likely to be relatively small because >90% of hovering metabolic rate is due to flight muscles (Suarez, 1992; Taylor,

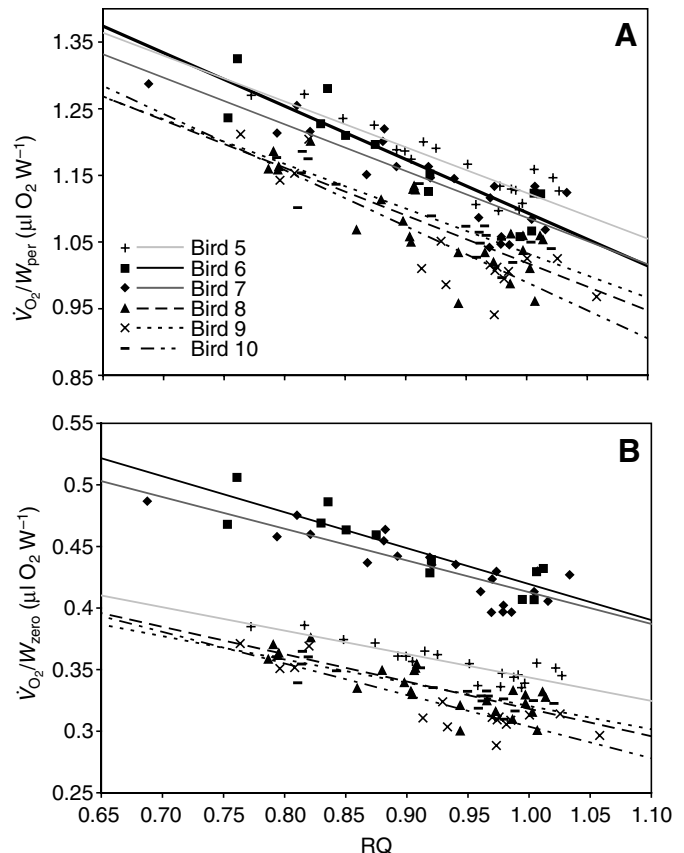


Fig. 3. Oxygen consumption rate per unit hovering power output ( $\dot{V}_{O_2}/W$ ) in relation to respiratory quotient (RQ) in rufous hummingbirds (*S. rufus*) assuming (A) perfect ( $P_{per}$ ) or (B) zero ( $P_{zero}$ ) elastic storage.

1987). In addition, Welch et al. show a remarkably strong correlation between the rise in RQ towards 1.0 and the approach of the carbon isotope signature of expired  $CO_2$  towards the value of recently ingested sugars (Welch et al., 2006), suggesting that the shift in RQ is due mainly to the increased oxidation of dietary sugar. Further, because there is oxygen consumption associated with lipogenesis, as hummingbirds shift from the fasted to the fed state and lipogenesis is activated, oxygen consumption should increase. Instead, we find that after we correct for slight differences in mechanical power output over the course of the experiment, oxygen consumption declines more than would be predicted solely by consideration of the proton stoichiometry associated with the metabolic substrate in use, not less.

Indeed, regardless of the assumption of perfect or zero elastic storage, the estimate of the difference in P/O ratio between glucose and palmitate oxidation for Anna's hummingbirds is significantly greater ( $P_{per}$ ,  $t_3=10.3333$ ,  $P=0.0019$ ;  $P_{zero}$ ,  $t_3=3.9294$ ,  $P=0.0293$ ) than the theoretical 14.9% difference estimated by Brand (Brand, 2005). The difference in P/O ratio between glucose and palmitate oxidation for rufous hummingbirds is significantly different from 14.9% when perfect elastic storage is assumed, but not when zero elastic

Table 2. Linear regression of  $O_2$  consumption rate per unit hovering power output ( $\dot{V}_{O_2}/W$ ), assuming either perfect ( $P_{per}$ ) or zero elastic storage ( $P_{zero}$ ), versus respiratory quotient (RQ) and predicted relative difference in  $\dot{V}_{O_2}/W$  between fat and carbohydrate metabolism

|                  | Bird no. | Linear regression of $\dot{V}_{O_2}/W$ versus RQ | $r^2$ | Predicted percentage difference* |
|------------------|----------|--|-------|----------------------------------|
| <i>C. anna</i>   |          |  |       |                                  |
| Using $P_{per}$  | 1        | =2.17–0.96×RQ                                    | 0.82  | 18.9                             |
|                  | 2        | =2.32–0.99×RQ                                    | 0.59  | 17.7                             |
|                  | 3        | =1.92–0.82×RQ                                    | 0.44  | 17.7                             |
|                  | 4        | =2.00–0.85×RQ                                    | 0.51  | 17.7                             |
|                  |          |  |       | Mean=18.0±0.6                    |
| Using $P_{zero}$ | 1        | =1.00–0.42×RQ                                    | 0.69  | 17.4                             |
|                  | 2        | =1.03–0.40×RQ                                    | 0.54  | 15.5                             |
|                  | 3        | =1.15–0.46×RQ                                    | 0.42  | 16.4                             |
|                  | 4        | =1.24–0.50×RQ                                    | 0.46  | 16.4                             |
|                  |          |  |       | Mean=16.4±0.8                    |
| <i>S. rufus</i>  |          |  |       |                                  |
| Using $P_{per}$  | 5        | =1.89–0.80×RQ                                    | 0.82  | 17.5                             |
|                  | 6        | =1.79–0.70×RQ                                    | 0.74  | 15.8                             |
|                  | 7        | =1.73–0.71×RQ                                    | 0.65  | 16.9                             |
|                  | 8        | =1.83–0.84×RQ                                    | 0.79  | 19.8                             |
|                  | 9        | =1.70–0.67×RQ                                    | 0.79  | 15.8                             |
|                  | 10       | =1.81–0.69×RQ                                    | 0.81  | 15.1                             |
|                  |          |  |       | Mean=16.8±1.7                    |
| Using $P_{zero}$ | 5        | =0.71–0.29×RQ                                    | 0.79  | 16.8                             |
|                  | 6        | =0.67–0.26×RQ                                    | 0.73  | 15.3                             |
|                  | 7        | =0.54–0.22×RQ                                    | 0.67  | 16.8                             |
|                  | 8        | =0.56–0.26×RQ                                    | 0.80  | 19.7                             |
|                  | 9        | =0.51–0.19×RQ                                    | 0.78  | 14.6                             |
|                  | 10       | =0.53–0.19×RQ                                    | 0.78  | 13.8                             |
|                  |          |  |       | Mean=16.2±2.1                    |

\*Predicted relative difference in  $\dot{V}_{O_2}/W$  between an RQ of 0.71 (fat) and 1.0 (carbohydrate).

storage is assumed ( $P_{per}$ ,  $t_5=2.7676$ ,  $P=0.0395$ ;  $P_{zero}$ ,  $t_5=1.4760$ ,  $P=0.2000$ ). These discrepancies may simply be due to estimation or measurement error. For the former, it should be noted that the power estimates for flight contain several assumptions that have yet to be evaluated, although we did use an empirically derived value for the profile drag coefficient (Altshuler et al., 2004a). However, the extremes of zero and perfect elastic energy storage almost certainly bracket the true values for mechanical power requirements. For the latter, it is worth noting that the difference in P/O ratio between glycogen and palmitate oxidation is 18.7% (Brand, 2005). How glucose and fructose are metabolized in hummingbirds prior to oxidation during hovering flight has not been established. However, mammalian studies reveal that dietary fructose is converted to glucose by the liver (Delarue et al., 1993); blood fructose concentration is low and skeletal muscles display low capacities for fructose oxidation (Kristiansen et al., 1997; Kristiansen et al., 1996). Irrespective of the details concerning the metabolism of ingested sucrose, our estimates fall within the range predicted by all possible scenarios and support the hypothesis that carbohydrate oxidation yields a higher P/O ratio

than fatty acid oxidation. As neither RQ values nor the current stable carbon isotope results allow discrimination between glucose and glycogen use, we plan to employ *in vivo*  $^{13}C$ -nmr spectroscopy to resolve this issue in future studies.

Another potential contributor to the slightly larger than expected difference in  $\dot{V}_{O_2}$  between carbohydrate and fatty acid oxidation might be the latter's activation of mitochondrial proton leak. In hummingbirds, a mitochondrial uncoupling protein (UCP) homologue has been found that is most abundant in flight muscles and, when expressed in yeast, stimulates mitochondrial proton leak (Vianna et al., 2001). Fatty acid oxidation results in production of reactive oxygen species (St-Pierre et al., 2002) that, in turn, activate avian UCP (Talbot et al., 2004). Thus, it is possible that some degree of mitochondrial uncoupling, leading to decreased P/O ratio, may occur during fatty acid-fueled hummingbird flight. Rolfe et al. (Rolfe et al., 1999) suggest that proton leak might be responsible for a non-trivial proportion of oxygen consumption in electrically stimulated rat leg muscles. However, the extent to which such futile cycling of protons leads to elevated oxygen consumption in hovering hummingbirds remains unknown.

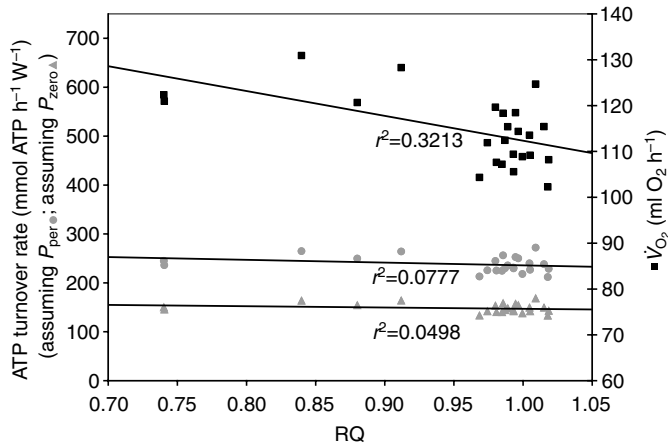


Fig. 4. The relationship between whole-animal oxygen consumption rate ( $\dot{V}_{O_2}$ ; ml  $O_2$   $h^{-1}$ ) and whole-animal ATP turnover rate per unit power output (mmol ATP  $h^{-1}$   $W^{-1}$ , assuming either perfect,  $P_{per}$ , or zero,  $P_{zero}$ , elastic storage) to respiratory quotient (RQ). The slope of whole-animal  $\dot{V}_{O_2}$  in relation to RQ is significantly different from zero ( $F_{1,22}=10.4134$ ,  $P=0.0039$ ). The slopes of whole-animal ATP turnover rate per unit power output in relation to RQ are not significantly different from zero (assuming  $P_{per}$ ,  $F_{1,22}=1.8537$ ,  $P=0.1871$ ; assuming  $P_{zero}$ ,  $F_{1,22}=1.1524$ ,  $P=0.2947$ ). Note that >90% of  $\dot{V}_{O_2}$  and ATP turnover rate during hovering are accounted for by flight muscles. Example using Anna's hummingbird (*C. anna*) number 3.

If whole-animal  $\dot{V}_{O_2}$  values reflect substrate-dependent differences in P/O ratios, then  $\dot{V}_{O_2}$  values can be used to estimate ATP turnover rates after properly correcting for the effect of metabolic substrate. Using Anna's hummingbird 3 as an example (Fig. 4), there is a significant negative relationship between whole-animal oxygen consumption rate and RQ ( $F_{1,22}=10.4134$ ,  $P=0.0039$ ). Thus, whole-body  $\dot{V}_{O_2}$  declines despite the gain in body mass as repeated feeding occurs and RQ climbs from 0.7 to 1.0. Upon correction for the effect of metabolic substrate on P/O ratio and for the effect of small differences in mass on mechanical power requirements, the resulting estimates of whole-animal ATP turnover rate per unit power output display no significant relationship to RQ (assuming  $P_{per}$ ,  $F_{1,22}=1.8537$ ,  $P=0.1871$ ; assuming  $P_{zero}$ ,  $F_{1,22}=1.1524$ ,  $P=0.2947$ ). Thus, the bird used a constant amount of ATP per unit mechanical power output during hovering as it went from the fasted to the fed state. This illustrates the utility of indirect calorimetry for the estimation of ATP turnover rate. In addition, the well-established stoichiometric relationships between moles of substrate oxidized and moles of  $O_2$  consumed allow straightforward estimates of the flux rates through catabolic pathways. Assuming that palmitate is the substrate oxidized while the RQ is 0.71, and based on the regression of oxygen consumption rate (ml  $O_2$   $h^{-1}$ ) as a function of RQ (Fig. 4), fatty acid oxidation would occur at a rate of  $4.14 \mu\text{mol min}^{-1}$  in bird no. 3 as it hovers in the fasted state. Assuming that glucose is oxidized when the RQ is 1.0, glycolytic flux (and glucose oxidation) would occur in this bird at a rate of  $13.93 \mu\text{mol min}^{-1}$  during hovering in the fed state. Rather than

being a flawed technique for the estimation of 'metabolic rates' in animals, we show here a set of circumstances in which indirect calorimetry serves as the method of choice.

Suarez and colleagues (Suarez et al., 1990; Suarez and Gass, 2002) proposed that hummingbirds maximize net energy gain by engaging in short foraging bouts while oxidizing dietary carbohydrate. Flying short distances for short durations while foraging results in reliance upon dietary sugar as the main oxidative substrate. This prevents the need to deplete fat stores but also avoids the inefficiency of investing energy into the synthesis of fat and then using fat to fuel foraging. Hochachka and colleagues (Hochachka et al., 1991; McClelland et al., 1998) hypothesized that a preference for carbohydrate oxidation, resulting in lower  $O_2$  requirements, may have evolved as an evolutionary adaptation to hypobaric hypoxia. Our evidence of substrate-dependent P/O ratios in hummingbirds and, in particular, the reduced requirement for  $O_2$  during carbohydrate oxidation provides support for Hochachka's hypothesis. Hummingbird flight performance has been repeatedly studied in physically variable gas mixtures, most commonly in normoxic heliox. In these density-reduction trials, hummingbirds compensate for increased flight requirements by substantially increasing the stroke amplitude of their wings and only moderately increasing the wingbeat frequency (Altshuler and Dudley, 2003; Chai and Dudley, 1995). This suggests that neuromuscular adjustments to increase stroke amplitude during a bout of hovering may incur lower muscle power expenditures than increases in contractile frequency. In oxygen-reduction experiments, hummingbirds accordingly decreased wingbeat frequency during sustained hovering, without a concomitant increase in stroke amplitude (Altshuler and Dudley, 2003). Substantial increases in wingbeat frequency have only been seen during transient load-lifting trials, which are brief and probably anaerobic (Altshuler and Dudley, 2003; Chai, 1997; Chai et al., 1997). These kinematic studies collectively suggest that oxygen delivery to flight muscle mitochondria may limit muscle performance during sustained flight (more than several seconds) at ecologically relevant elevations. Many species of hummingbirds have been observed to forage at high elevation (Altshuler, 2006; Altshuler et al., 2004b). Under these conditions, significant benefits to hummingbird flight performance would be gained through the  $O_2$ -sparing effect of carbohydrate oxidation. The combined benefits of oxidizing carbohydrate qualify it as a premium fuel for hummingbird flight.

#### List of symbols and abbreviations

|                  |  |
|------------------|--|
| $\dot{V}_{O_2}$  | rate of oxygen consumption (ml $O_2$ $g^{-1}$ $h^{-1}$ )                                     |
| $\dot{V}_{CO_2}$ | rate of carbon dioxide production (ml $CO_2$ $g^{-1}$ $h^{-1}$ )                             |
| RQ               | respiratory quotient ( $=\dot{V}_{CO_2}/\dot{V}_{O_2}$ )                                     |
| $M_b$            | body mass (g)  |
| $P_{per}$        | Total hovering power requirement assuming perfect elastic storage of energy ( $W$ $g^{-1}$ ) |
| $P_{zero}$       | Total hovering power requirement assuming zero elastic storage of energy ( $W$ $g^{-1}$ )    |

|                  |   |
|------------------|---|
| $P_{\text{pro}}$ | profile power; the energy required to overcome profile drag forces on the wings ( $\text{W g}^{-1}$ )                             |
| $P_{\text{acc}}$ | inertial power; the energy necessary to accelerate the wings during the first half of a half-stroke ( $\text{W g}^{-1}$ )         |
| $P_{\text{ind}}$ | induced power; the energy required to move sufficient air in order to overcome the downward pull of gravity ( $\text{W g}^{-1}$ ) |

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