The power of feeder-mask respirometry as a method for examining hummingbird energetics ☆

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ABSTRACT

Many birds spend important portions of their time and energy flying. For this reason, quantification of metabolic rates during flight is of crucial importance to understanding avian energy balance. Measurement of organismal gas exchange rates using a mask enclosing the whole head or respiratory orifices has served as an important tool for studying animal energetics because it can free the rest of the body, permitting movement. Application of so-called "mask respirometry" to the study of avian forward flight energetics presents unique challenges because birds must be tethered to gas analysis equipment thus typically necessitating use of a wind tunnel. Resulting potential alterations to a study organism's behaviour, physiology, and aerodynamics have made interpretation of such studies contentious. In contrast, the study of hovering flight energetics in hummingbirds using a specialized form of mask respirometry is comparatively easy and can be done without a wind tunnel. Small size, hovering flight, and a nectarivorous diet are characteristics shared by all hummingbird species that make these birds ideally suited for this approach. Specifically, nectar feeders are modified to function as respirometry masks hummingbirds voluntarily respire into when hover-feeding. Feeder-mask based respirometry has revealed some of the highest vertebrate metabolic rates in hovering hummingbirds. In this review I discuss techniques for the successful measurement of metabolic rate using feeder-mask respirometry. I also emphasize how this technique has been used to address fundamental questions regarding avian flight energetics such as capacities for fuel use and mechanisms by which ecology, behaviour and energy balance are linked.

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1. Introduction: the history and development of feeder-mask based respirometry in hummingbirds

The study of avian energetics often centers on this group's hallmark form of locomotion: powered flight. Flight enables birds to travel farther and faster than comparably sized terrestrial or aquatic species. Biologists have long sought to quantify the energetics of powered flight because such information is crucial to understanding associated underlying specializations in avian physiology and morphology, as well as how this mode of locomotion relates to nearly every aspect of bird ecology, behaviour and evolution. However, it is inherently challenging to effectively focus equipment on a bird moving through the air. Thus, accurate quantification of the energetics of bird flight has been among the more challenging endeavours in all of vertebrate organismal biology.

Lavoisier first noted that oxygen (O_2) consumption could be used to predict heat production in animals in the latter half of the 18th century. With the subsequent development of increasingly accurate gas analysis and flow rate measurement equipment and associated techniques, respirometry, the study of rates of gas exchange, has been confirmed as a reliable means of indirectly quantifying organismal heat production (calorimetry) and thus metabolic rate. Respirometry, in various forms, has been used in the study of avian energetics for more than two centuries. Lavoisier himself included observations of a sparrow in his important studies on animal respiration (Lavoisier, 1777). Following Lavoisier's observations, respirometry was applied to the study of resting metabolism in birds beginning in the 1820s. Systematic examination of the relationship between resting or basal metabolic rate and body mass in birds began in the early 20th century (summarized in Benedict and Fox, 1927; Lasiewski and Dawson, 1967; Kleiber, 1975). In all such studies, however, gas exchange was measured on resting or relatively inactive birds placed entirely inside airtight containers. In this way, "box", "jar", or "chamber" respirometry, as this technique is variously known, is similar to traditional method of direct calorimetry, which requires the confinement of organisms inside measurement apparatus (see Lighton et al., this issue). In most instances, this confinement precluded the study of metabolic rate during locomotion or exercise (Lighton et al., this

issue). As a result studies examining the energetics of flight remained beyond the reach of either approach.

Respirometry was first used to examine avian flight energetics when, in the late 1940s, Oliver Pearson recognized that the hummingbirds' ability to sustain hovering flight within confined spaces made them uniquely suited for study using existing chamber-based techniques. Pearson (1950) placed hummingbirds in small, completely sealed bell jars and determined the rate of O_2 consumption ($\dot{V}_{O_2}$) during rest, torpor, and, importantly, during hovering flight using a closed-circuit, constant-pressure, volumetric technique (this technique reviewed more completely in Lighton et al., this issue). Layers of soda lime and calcium carbonate were placed at the bottom of the jar to absorb the carbon dioxide (CO_2) and water from the air. As the bird respired and consumed O_2 the volume of gas within the jar decreased at a measurable rate related to its activity state. Pearson's important work heralded a breakthrough in the study of avian energetics, the metabolic scope of exercise, and of the limits to vertebrate metabolism. Other researchers, including Robert Lasiewski, subsequently employed similar closed-circuit system designs in the determination of the metabolic rates of hovering hummingbirds (e.g. Lasiewski, 1963; Epting, 1980). The fact that subsequent studies on hovering hummingbirds using other techniques (see below) have recorded metabolic rates generally comparable to those reported by Lasiewski (1963) is a testament to the potential accuracy of this approach. However, the closed-circuit techniques employed by Pearson and Lasiewski were subject to important limitations and technical challenges. The decreasing oxygen concentration inside the chamber represented a potentially deleterious physiological challenge for the study organism, though Pearson dismissed this idea claiming there was "no indication of oxygen debt" following trials (Pearson, 1950). In addition, accurate determination of hovering metabolic rate was hampered when hummingbirds failed to hover for extended durations. Lasiewski's most confident measurements came from a juvenile Costa's hummingbird (*Calypte costae*) that hovered continuously for 50 min (Lasiewski, 1963), while Epting only included measurements from hummingbirds that sustained hovering for 2–5 min (Epting, 1980).

With the increasing availability of sensitive gas analyzers in the mid twentieth century, closed-circuit respirometry was generally abandoned in favor of open-circuit, flow-through respirometry for the study of avian energetics. Like in Pearson's pioneering work, the first applications of this respirometric approach to the study of metabolic rate during flight involved placing hummingbirds in small containers (e.g. Wolf and Hainsworth, 1971; Schuchmann, 1979). Unlike in Pearson's studies however, ambient air was continuously drawn through the chamber before being passed through the gas analyzers (this technique reviewed more completely in Lighton et al., this issue). The study of avian flight energetics using this flow-through respirometry technique was, as with Pearson's closed-circuit approach, limited to hummingbirds that could hover in the necessarily small chambers. Advantages over the closed-circuit, constant-pressure approach included limited danger of hypoxia and hypercapnia (provided gas flow rates were sufficiently high; see below). Nonetheless, accurate measurement of hovering metabolic rate was still only possible when birds sustained hovering flight for significant durations. Relatively large chamber volumes (≈ 10 L) and low flow rates (<600 ml/min) meant steady state rates of oxygen consumption were only reached 15–50 min after the initiation of data collection (Wolf and Hainsworth, 1971).

The study of avian energetics during forward flight advanced greatly when Vance Tucker first utilized affixed-mask respirometry to study the energetics of bird flight (Tucker, 1966, 1968). Specifically, Tucker attached vacuum-formed, celluloid masks to budgerigars (*Melopsittacus undulatus*) trained to fly in a wind tunnel (Fig. 1; Tucker, 1966, 1968). While birds flew steadily at one of the range of velocities in the wind tunnel, air was drawn at a constant rate through the mask and via attached tubing to gas analyzers by a downstream pump. Following in
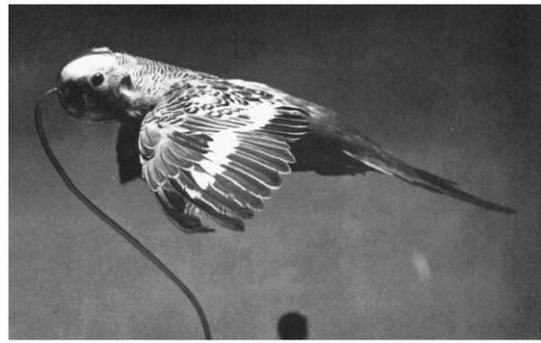

Fig. 1. A budgerigar (*Melopsittacus undulatus*), trained by V. A. Tucker to wear a respirometry mask, flying within a wind tunnel at ≈ 35 km/h (Tucker, 1968). This and Tucker's lesser known 1966 study (Tucker, 1966) were the first applications of affixed-mask respirometry within a wind tunnel to the study of the energetics of forward flight in birds.

the footsteps of Tucker's pioneering studies, researchers have used variations of this affixed-mask respirometric technique to study the energetic cost of avian flight in relation to flight velocity (e.g. Tucker, 1968, 1972; Hudson and Bernstein, 1983; Ward et al., 2001, 2002, 2004; Bundle et al., 2007), the effects of variation in angle of ascent or descent (e.g. Tucker, 1968, 1972; Bernstein et al., 1973; Hudson and Bernstein, 1983), and variation in mass and other morphological characteristics within and among individuals of a species (e.g. Butler et al., 1977) or between species (e.g. Bernstein et al., 1973; Bundle et al., 2007).

The use of a mask enclosing only the head or respiratory orifices of a study organism offers important advantages over chamber-based, flow-through respirometry for the study of flight energetics. Any bird that can be trained to fly in a wind tunnel can be studied. Further, metabolic rate can be determined over much shorter time scales. Bird flight within wind tunnels represent an imperfect simulation of natural flight because rather than the bird moving through more or less still air, air instead moves past a more or less still bird. While turbulence can be minimized by appropriate tunnel design, moving air within a tunnel will be more turbulent than still air through which a free flying bird moves. While not a perfect simulation, wind tunnels remain one of the most useful tools for studying forward flight (Rayner, 1994). To optimize accurate approximation of natural forward flight in the wild, the working section of a wind tunnel should ideally be large enough so that a bird can fly within an area far enough from any tunnel surface so that boundary effects, interactions between the vortices caused by the wing and the tunnel walls, are minimized (Rayner, 1994, 1999). This means that the volume of air contained within close-circuit wind tunnels (including areas upstream and downstream of the working area) is typically so large that $\dot{V}_{O_2}$ and $\dot{V}_{CO_2}$ cannot accurately be determined by sampling tunnel air directly (but see Torre-Bueno and Larochelle, 1978). Instead, capture of expired breath in a mask allows sampling of expired gases more directly from their point of origin while avoiding their dilution in a larger chamber.

While it has enabled great advances in the study of forward flight energetics, affixed-mask respirometry on birds in wind tunnels is subject to some disadvantages. It can be difficult to elicit the desired behaviour from a bird while it wears a mask. Tucker noted that while one of the two budgerigars studied flew with apparent disregard for the mask it was wearing, "the other learned to remove the mask with its feet while in flight...indulging in this exasperating habit" sooner if compelled to expend energy at greater rates, such as during inclined flight (Tucker, 1968). Further, the additional weight and drag imposed on a bird wearing a mask and tubing as well as boundary effects within a wind tunnel complicates the study of the energetics of forward flight (Rayner, 1994). Experimentally and mathematically derived estimates of the increase in the metabolic cost of forward flight due to mask and tubing drag and boundary effects at roughly 10% and 30%, respectively (Tucker, 1968; Bernstein et al., 1973;
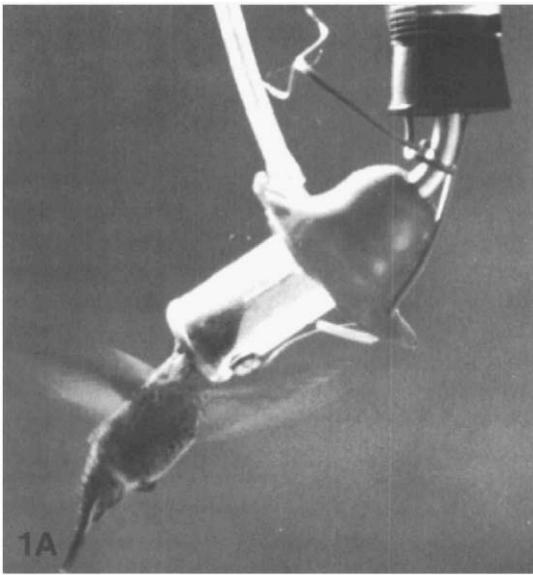
Fig. 2. An Anna's hummingbird (*Calypte anna*) hover-feeding at a feeder contained inside a plastic tube arranged to function as a mask (Bartholomew and Lighton, 1986). This was the first application of mask respirometry to the study of avian energetics in wild animals and illustrates both this advantage of mask respirometry as well as the readiness with which hummingbirds will 'voluntarily' wear the mask.

imposed by the mask are rigorously accounted for using, for example, force plates to directly measure drag on mounts of stuffed birds placed within the tunnel (Tucker, 1972; Bernstein et al., 1973; Bernstein, 1976), variation in the position of birds during the course of active flight can lead to dynamic and unpredictable effects on the magnitude of this drag (Rayner, 1994). In addition, birds tethered to gas analyzers via tubing are not free to move in all directions, limiting study to steady state forward flight only.

As noted above, the ability of hummingbirds to hover made them the perfect choice for pioneering studies on the energetics of flight using chamber-based respirometric approaches (Pearson, 1950; Lasiewski, 1963). Following the first applications of affixed-mask respirometry to the study of forward flight energetics in other birds, Martin Berger adapted hummingbird feeders for use in respirometry by securing a plastic mask around the feeder opening so that hungry hummingbirds voluntarily donned the mask as they inserted their heads to feed (Berger and Hart, 1972; Berger, 1974). Examples of this type of respirometry setup are shown in Fig. 2–4 (with the schematic originally presented by Berger and Hart shown in the inset of Fig. 4). As with other applications of mask respirometry, this system operated by pulling air past the birds' head and through the mask and attached tubing leading away from the feeder to gas analyzers. However, this approach offers some distinct advantages over affixed-mask respi-rometry. Importantly, the hummingbird does not have to support the weight of the mask. In addition, the researcher can dictate the position of the feeder-mask, and thus the feeding hummingbird, within the flight arena or wind tunnel. Boundary effects and the effects of drag on air tubing leading from the mask can be minimized or avoided by careful positioning of the feeder-mask. Recently, researchers have employed feeder-mask respirometry to quantify metabolic rate during flight as a function of velocity in Anna's (*Calypte anna*), Allen's (*Selasphorus sasin*), and rufous (*Selasphorus rufus*) hummingbirds (Clark and Dudley, 2009, 2010; Tobalske, 2009). In contrast to
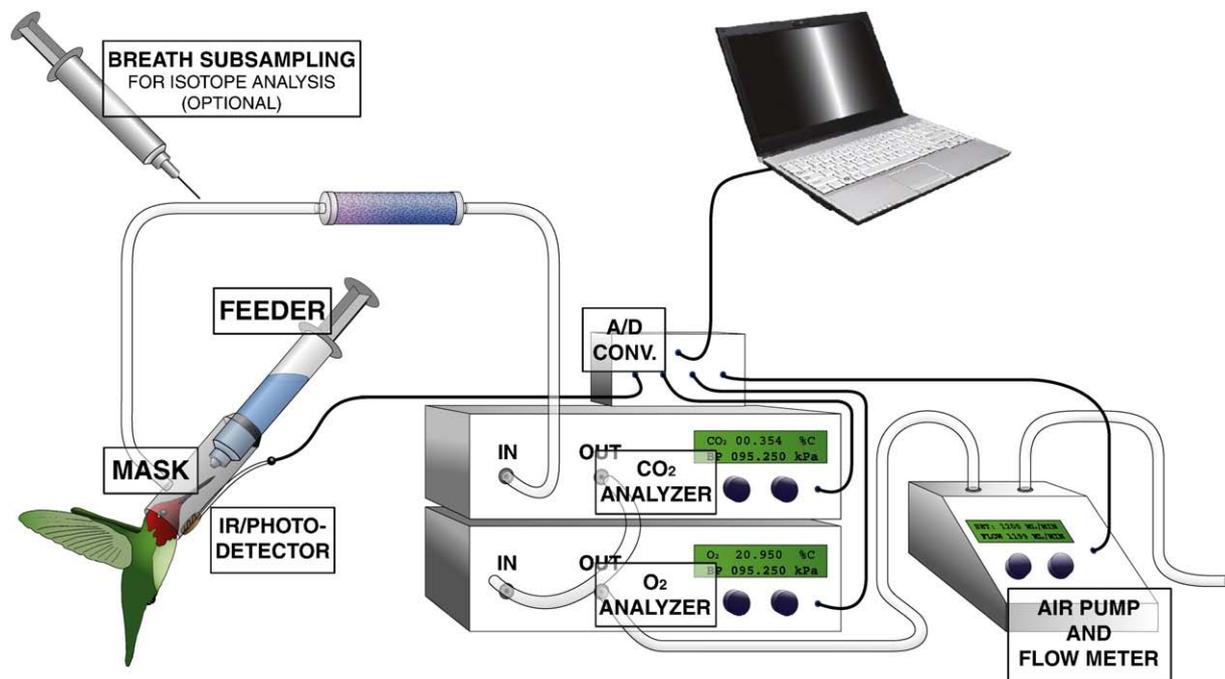
Rayner, 1994), indicating the importance of accounting for these phenomena. Corrections for these effects on the aerodynamic and energetic cost of flight have been attempted, beginning with Tucker's study (1972) of the laughing gull (*Leucophaeus atricilla*). Subsequent-ly, other researchers have produced similar corrections (Bernstein et al., 1973; Bernstein, 1976). However, even when the effects of drag

Fig. 3. Example setup for performing feeder-mask respirometry on a hover-feeding hummingbird. This setup employs gas analysis of the main airstream and is therefore suitable for use with relatively low flow rates only. Breath subsampling upstream of chemical scrubbing of CO_2 is optional (for carbon isotopic tracking, etc.). If performed, such subsampling would alter calculated rates of oxygen consumption or carbon dioxide production, and is therefore best done during parallel trials. Drying of the airstream with chemical scrubbers (e.g. Drierite) is shown. As both oxygen AND carbon dioxide levels are measured in this example, no scrubbing of CO_2 is necessary before air enters the oxygen analyzer. The IR/photodetector records the presence of the hummingbirds head within the mask. Integration of total volumes of oxygen consumed or carbon dioxide produced by the hummingbird while in the mask over the duration of time its head was present permits calculation of 'instantaneous' rates of oxygen consumption or carbon dioxide production even when levels of neither gas reach a steady state value during any one feeding sally.
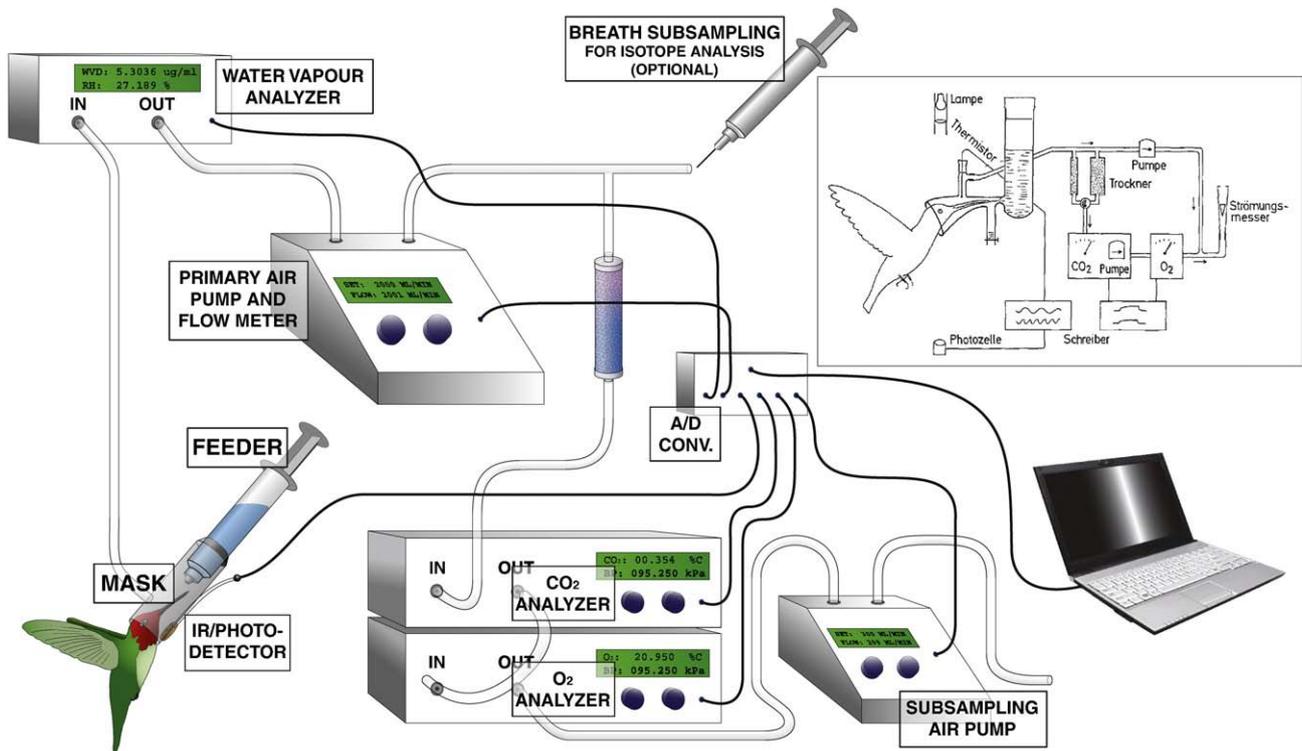
Fig. 4. Example setup for performing feeder-mask respirometry on a hover-feeding hummingbird. This setup analyzes air subsampled from the main airstream. Flow rates excurrent from the mask can be higher than in the setup shown in Fig. 3 while flow rate of the subsampled airstream is kept relatively low. In this case, additional subsampling of the airstream for the purposes of stable isotope tracking of expired breath, etc., can be done at the point indicated with no effect on V_{O_2} and V_{CO_2} measurements. Note: A schematic of the original setup published by Berger and Hart (1972) is shown in the inset for comparison.

Berger's notable previous study on the subject in which affixed-mask respirometry was employed, these more recent studies have shown that metabolic rate when flying at moderate speeds (<10 m/s) is lower than during hovering. It should be noted that feeder-mask respirometry carries with it an important limitation when used within a wind tunnel. The stationary feeder-mask necessarily sits just upstream of the hummingbird. Air flow around the feeder-mask is altered (Fig. 5) and the precise effects this has on airflow around the
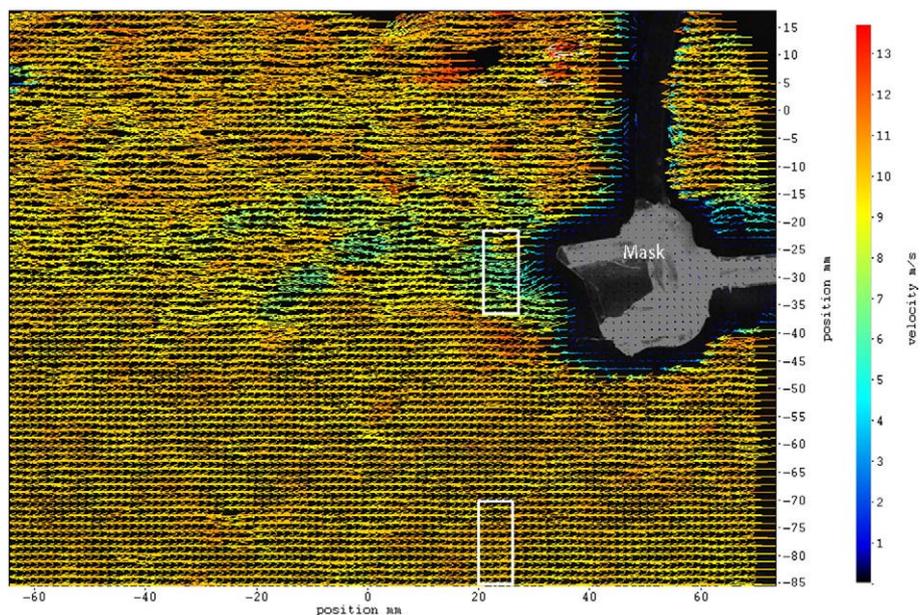

Fig. 5. Particle image velocimetry (PIV) of a vertical sheet of air flow bisecting the long axis of a respirometry feeder-mask suspended within a wind tunnel. The mean velocity of air flow in the operating section of the wind tunnel was 10 m/min in this instance. The drop in air velocity and increase in turbulence just downstream of the mask, indicated by the blue/green, non-uniformly directed arrows, occurs at the point where the hummingbird's body would be positioned during feeding. This turbulence would result in unpredictably altered aerodynamics during forward flight. This represents an unavoidable complication when performing respirometric measurements on birds in wind tunnels using a stationary mask. Image from Clark and Dudley (2009; supplemental online materials).

hummingbird, imposed drag, and thus metabolic rate during forward flight remain unclear.

The hummingbird's desire to obtain the nectar within the feeder-mask ensures their cooperation when obtaining respirometric measurements. The observation that free-ranging hummingbirds will voluntarily insert their heads into the mask at feeders placed outdoors supports this assertion (Fig. 2; Bartholomew and Lighton, 1986; K. Welch, pers. observ.). The portability of the feeder-mask respirometry setup and the ease with which it can be applied under field conditions (e.g. Bartholomew and Lighton, 1986; Welch and Suarez, 2008) is another advantage. The ability to deploy a feeder-mask respirometry setup either within a flight arena in the lab, or in various field locations, has facilitated better understanding of the effects environmental parameters have on the cost of hovering flight. Replacement of ambient flight arena air with variable air mixtures has allowed researchers to understand how the costs of flight vary with oxygen availability and air density (Chai and Dudley, 1995, 1996; Chai, 1997). Other studies have explored the relationship between hovering metabolic rate and elevation through laboratory simulations in a hypobaric chamber (Berger, 1974) or by repeated trials at multiple elevations in the field (Welch and Suarez, 2008). Additionally, several groups have explored how temperature affects hovering metabolic rate in these small vertebrate endotherms in both the laboratory (Berger and Hart, 1972; Chai et al., 1998; Evangelista et al., 2010) and the field (Welch and Suarez, 2008). Hummingbirds will perform virtually any flight behaviour such as pure yaw turns and sideslip flight as they track a moving feeder (pers. observ.; D. L. Altshuler, pers. comm.). By converting the feeder into a feeder-mask, measurement of metabolic rate is therefore possible on a wider range of flight behaviours in hummingbirds than in any other avian group. Though sustained hovering flight is a behaviour not exhibited by other birds, these studies have improved the general understanding of how such environmental parameters impact flight in all avian groups and flying vertebrates in general.

While feeder-mask respirometry offers the advantage of eliciting sustained, steady, voluntary flight, it is impossible to separate the cost of flight from the energetic costs associated with imbibing nectar (Bartholomew and Lighton, 1986). Given the flight muscles account for such a large proportion of hummingbird body mass (Suarez et al., 1986; Wells, 1993), and the fact that estimates of the metabolic cost of flight made using mathematical modeling of associated aerodynamic forces on free flying hummingbirds are so great (e.g. Epting, 1980; Wells, 1993; Chai and Dudley, 1995, 1996; Chai et al., 1998; Altshuler et al., 2004; Welch and Suarez, 2007), it's likely that the costs of imbibing nectar can be largely ignored (Suarez, 1992). Regardless, hover-feeding at a mask quite faithfully replicates a behaviour in which hummingbirds engage for a substantial portion of their day (Stiles, 1971; Wolf and Hainsworth, 1971). Thus, data generated utilizing the feeder-mask respirometric technique are of great importance in improving understanding of hummingbird energetics in particular, and avian energetics more generally.

2. Methodological standards and potential technical issues in feeder-mask respirometry

With either an attached or feeder-mask, the size of the effective respiration chamber employed is reduced so that it encloses only the head or respiratory orifices. Less air space within the whole of the respirometry system (including airlines to and from a chamber or mask and within various pieces of equipment) means a faster response time and greater change in gas levels relative to analyzer noise. Further, because masks can often be made so small that the effective volume of air enclosed within them (when worn) may be less than the tidal volume respired by the study organism, the danger of accumulation of hypoxic or hypercapnic air for inhalation is greatly reduced. Rather, it is the loss of respired gases around the edges of the

mask that is a greater concern. Just as with chamber-based respirometry, a critical flow rate exists below which calculated $\dot{V}_{O_2}$ and $\dot{V}_{CO_2}$ values will be underestimates of the actual values (McNab, 2006). However, in affixed or feeder-mask respirometry this is because sub-critical flow rates fail to capture all expired gases in the mask, while sub-critical flow rates lead to inadequate mixing of air in a chamber (McNab, 2006). Because mask sizes and shapes, as well as the aerodynamic environments around birds are unique to each given study, there is no simple rule to identifying flow rates sufficiently high to capture all expired air through the mask while sufficiently low as to lead to differences in O_2/CO_2 levels in the excurrent air stream large enough to accurately detect against analyzer noise and drift. Typical approaches for identifying appropriate flow rates involve consideration of animal metabolic rate and size, and helpful calculators are available online (for example, see http://www.respirometry.org). Empirical determination of minimally appropriate flow rates can be done by introducing purified nitrogen (or another standard laboratory gas) into the mask at a known low flow rate while the speed of air flowed through the wind tunnel is varied. When all of the introduced nitrogen is captured by the mask, the apparent O_2 and CO_2 levels in the excurrent airstream will be lowered by a predictable amount. Equations presented below can be used to predict the degree of O_2 and CO_2 dilution as a function of flow rate. In addition to allowing quantification of system washout kinetics, insufficient flow rates can be identified by artificially low apparent dilutions of the O_2 and CO_2 concentrations in the captured airstream. Alternatively, some researchers have attempted to empirically identify minimally appropriate flow rates via repeated respirometric measurements at progressively increasing flow rates (e.g. Tucker, 1968 when using an attached mask; Clark and Dudley, 2009 when using a feeder-mask). As flow rate is increased, apparent $\dot{V}_{O_2}$ and $\dot{V}_{CO_2}$ increase because more of the gases expired by the animal are captured. The flow rate above which apparent $\dot{V}_{O_2}$ and $\dot{V}_{CO_2}$ no longer vary significantly represents a minimum appropriate flow rate for respirometric measurements. An example of such a relationship, generated by Clark and Dudley (2009) as an Anna's hummingbird (C. anna) fed at a mask modified as a nectar feeder (see more on this below) while flying at 10 m/s in a wind tunnel, is shown in Fig. 6. The flow rate above which $\dot{V}_{O_2}$ no longer rises is approximately 3 L/min in this case and the researchers subsequently utilized a flow rate of 3.9 L/min during data collection
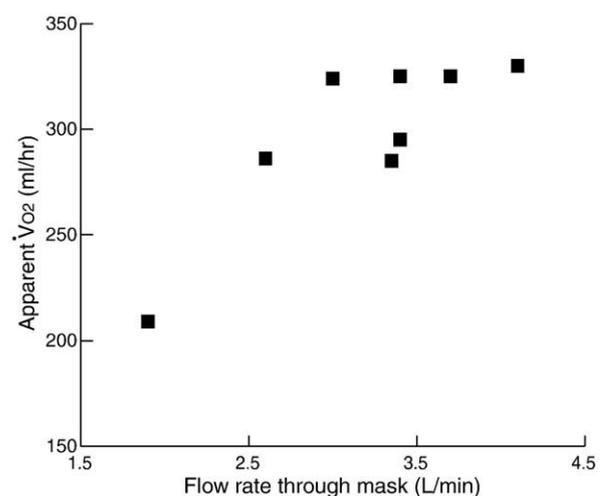

Fig. 6. Apparent oxygen consumption rate (V_{O_2}) as a function of flow rate from a feeder-mask. Data obtained from an Anna's hummingbird (*Calypte anna*) flying in a wind tunnel at 10 m/s. At low flow rates (<3 L/min), gases expired by the hummingbird are lost around the edges of the mask in part because of the high degree of turbulence in the wind tunnel, resulting in an underestimation of V_{O_2}. A flow rate of 3 L/min represents the critical value above which lost gases are minimized and accurate V_{O_2} values are obtained under these particular experimental conditions. Provided by Chris Clark from preliminary data for Clark and Dudley (2009).

(Clark and Dudley, 2009). Each of the two approaches described above has its own merits. Identification of minimal appropriate flow rates via introduction of nitrogen into the mask offers a controlled approach that will not suffer from undesirable variability in hummingbird mask visitation behaviour. On the other hand, because flow rates should be chosen with the expectation that hummingbird mask visitation behaviour will vary an approach to minimal appropriate flow rate selection involving actual hummingbird feeder-mask visitations while flow rates are sequentially varied can provide additional valuable insight.

Derivation of changes in O_2 and CO_2 levels in an airline flowing past an animal, and thus accurate respirometric measurement of animal $\dot{V}_{O_2}$ and $\dot{V}_{CO_2}$ requires knowledge of the respective fractional concentrations of all gases in both the incurrent and excurrent air as well as the flow rate. In many cases, the incurrent stream is ambient air which typically contains known and relatively constant fractional concentrations of component gases (e.g. ≈ 0.2095 for O_2). However, because feeder-mask respirometry can be accomplished within modestly sized chambers, replacement of ambient air with variable gas mixtures is feasible (Chai and Dudley, 1995, 1996; Altshuler et al., 2001). In such cases, baseline fractional concentrations of gases in the incurrent air may not readily be assumed. Fortunately, it is easy to measure these values by streaming air through the feeder-mask when the hummingbird is not present (called baselining). Typically, air is pulled through feeder-mask systems constantly throughout an extended period of data collection. Intermittent feeding sallies at the feeder-mask are bracketed by these periods of baseline data acquisition. Changes in fractional concentrations of O_2 and CO_2 resulting from a feeding are always referenced against these baseline values.

It is only possible to generate a constant and controlled airflow from downstream of a mask (pull-through respirometry) and this is also where flow rate is measured. Inaccurate flow rate measurement is typically the greatest potential source of error in the measurement of metabolic rate via flow-through respirometry. Accurate flow rate measurement is inherently technically challenging, but is of critical importance. It is possible to accurately quantify $\dot{V}_{O_2}$ and $\dot{V}_{CO_2}$ without necessarily measuring all four major component gases (O_2, CO_2, nitrogen, and water vapour) in the incurrent and excurrent air-streams. Because respiring animals do not alter nitrogen levels in the air, measurement of this component gas is typically ignored completely. The same holds true for helium and is relevant in experiments employing ambient air mixture replacement (e.g. with O_2–helium mixtures; Chai and Dudley, 1995, 1996). Water vapour and CO_2 levels in the air entering the mask may vary significantly due to varying local humidity and the activity of machinery and researchers nearby. Levels of these gases will also certainly vary in the excurrent air as respiring animals both produce CO_2 (a variable of interest here) and humidify air as a part of normal respiratory function. The addition of water vapour meters and CO_2 analyzers to any respirometry system is advisable both because accurate $\dot{V}_{O_2}$ calculation depends on knowing the effects these gases have on the overall composition of excurrent air and because they are physiologically relevant in their own right. Correction for the effect of water vapour on flow rate of air from the mask can be easily accomplished by applying Eq. (1.4) in the appendix (from a technique described in Lighton, 2008). This equation also requires quantification of barometric pressure and many water vapour meters or other gas analyzers already track this variable. However, when fractional concentrations of either CO_2 or water vapour are not measured they should ideally be chemically scrubbed before flow rate measurement occurs (e.g. Fig. 3). When relatively high flow rates are used (>2 L/min) it may be impractical to chemically scrub the main flow of the excurrent air of either water vapour or CO_2 because scrubber consumption becomes excessive. At the same time, passing of air through gas analyzers at these high flow rates should be avoided for several reasons.

Excessively high flow rates can damage paramagnetic oxygen analyzers, disrupt zirconium-cell oxygen sensors by promoting excess cooling of the sensor element, and excessively alter air pressure within equipment and tubing (a component of which is oxygen partial pressure, a variable of interest; Lighton, 2008). Nonetheless, as stated above, high flow rates through the respirometry mask may be necessary, particularly when the potential for loss of expired breath is a definite possibility due to turbulent air flow around the mask, such as in a wind tunnel. In such cases, subsampling of the airstream excurrent from the mask for gas analysis is the preferred approach (e.g. Fig. 4). Subsampling of excurrent air was employed during the first application of affixed-mask respirometry to the study of avian energetics (Tucker, 1966, 1968) and in many subsequent studies, including feeder-mask respirometric studies involving hummingbirds (e.g. Tucker, 1966, 1968, 1972; Berger and Hart, 1972; Bernstein et al., 1973; Berger, 1974; Butler et al., 1977; Epting, 1980; Gessaman, 1980; Hudson and Bernstein, 1981, 1983; Rothe et al., 1987; Wells, 1993; Chai and Dudley, 1995, 1996; Chai et al., 1996, 1998; Butler, 2000; Ward et al., 2002, 2004; Bundle et al., 2007; Clark and Dudley, 2009). In these cases, measurement of the main excurrent flow rate remains crucial. Because only the fractional concentration of gases in the subsampled air needs to be quantified it is not necessary to know the exact flow rate of this airstream, provided it is reliably constant and gas analyzers have been appropriately calibrated according to manufacturer specifications. However, when chemical scrubbing of the air occurs in the subsampled airstream but not the main excurrent flow, levels of these gases in air upstream of the scrubbers should be quantified in order to allow for correction of the main excurrent flow rate, at least in the case of water vapour. Provided flow rates are sufficiently high, levels of CO_2 in ambient and excurrent air will be low enough that failure to correct for its presence will not introduce substantial error into calculation of $\dot{V}_{O_2}$. However, failure to correct for the presence of water vapour can lead to overestimation of flow rates relative to a dried state by 1% or more under typical laboratory conditions (Lighton, 2008).

In some cases, it may be desirable to sample expired breath from a bird while it is flying, such as in studies tracking the stable isotopic signature of carbon in the exhaled CO_2 (e.g. Welch et al., 2006; Welch and Suarez, 2007). If gas samples are removed upstream of gas analyzers (Fig. 3), accurate measurement of $\dot{V}_{O_2}$ and $\dot{V}_{CO_2}$ is not possible. Provided mixing of air within the system is sufficiently high, determination of rates of $\dot{V}_{O_2}$ and $\dot{V}_{CO_2}$ relative to each other is still possible, permitting calculation of respiratory quotient or respiratory exchange ratio (RQ or RER = $\dot{V}_{CO_2}/\dot{V}_{O_2}$). However, when there is subsampling of the main excurrent air flow as in Fig. 4, breath samples may be taken from the main airstream downstream of all equipment with no effect on absolute $\dot{V}_{O_2}$ and $\dot{V}_{CO_2}$ calculation.

Various equations for calculating $\dot{V}_{O_2}$ and $\dot{V}_{CO_2}$ that commonly apply in feeder-mask respirometric setups when the various component gases are alternatively measured or scrubbed are presented in the appendix. It is important to note that the brief duration of feeding events characteristic of hummingbirds at a feeder-mask often results in gas fractional concentration recordings that are very dynamic. Because of their small size and high hovering metabolic rate, steady state $\dot{V}_{O_2}$ and $\dot{V}_{CO_2}$ values are rapidly reached following the transition from rest (perching) to exercise. The time it takes a hummingbird to fly from a nearby perch to the feeder-mask to feed is typically enough to ensure $\dot{V}_{O_2}$ and $\dot{V}_{CO_2}$ have reached steady state. However, because the air volume contained within the tubing and equipment comprising even a feeder-mask respirometry system cannot be zero, mixing of ambient air and gases expired by the bird will occur within the system and there will not be an instantaneous change in fractional concentrations of component gases, measured by gas analyzers, to the steady state levels associated with flight when the bird inserts its head to drink. Instead, mixing of pure ambient air both upstream and downstream of the bolus of ambient-plus-expired

air captured by the mask during the feeding sally results in volumetric washout. Particularly short feeding sallies may result in O_2 or CO_2 fractional concentration traces that appear to never reach a steady state value. Feeding sallies that are comprised of several repeated head insertions, rather than one or a few sustained insertions, will also generally increase the likelihood of dynamic and variable fractional gas concentration recordings. When a hummingbird feeds at the mask for relatively long durations, equilibrium fractional concentrations can be measured and an instantaneous $\dot{V}_{O_2}$ and $\dot{V}_{CO_2}$ can be calculated. Researchers adopting this strategy for calculation of $\dot{V}_{O_2}$ and $\dot{V}_{CO_2}$ simply exclude from calculation those feeding events during which equilibrium gas values were not reached (e.g. Chai and Dudley, 1995, 1996; Chai et al., 1998; Clark and Dudley, 2009, 2010). Depending upon flow rate, tubing length, and the type of gas analyzer used, uninterrupted feeding sallies as short as 2–3 s may yield gas fractional concentration traces that reach steady state values (Clark and Dudley, 2009), though values of 10–20 s may be characteristic of many other systems (pers. observ.). Respirometry system response kinetics can be quantified by the introduction of a steady flow of nitrogen gas into the mask. These results can then be used to establish minimum feeding durations that will yield steady state gas traces. However, when flow rate is too low or feeding sally durations are too short or are characterized by repeated head removals so that obvious equilibrium fractional concentrations of O_2 and CO_2 are not regularly observed it is still possible to derive accurate instantaneous $\dot{V}_{O_2}$ and $\dot{V}_{CO_2}$ estimates. There are feeding events of such short duration, or involving such dynamic head insertion behaviour that reliable estimates of metabolic rate are impossible. Feeding events of less than a second in duration, or for which the ratio of total feeding duration to head insertion events is less than one should almost certainly be excluded from analysis. Several approaches exist for deriving instantaneous $\dot{V}_{O_2}$ or $\dot{V}_{CO_2}$. Some, such as application of the Woakes instantaneous equation described in Woakes and Butler (1983), and summarized by Halsey et al. (2009), have not been applied to feeder-mask respirometry in published studies. Readers are referred to these papers for a more complete description of this approach. Two general approaches have been used throughout published studies employing feeder-mask respirometry on hummingbirds: the instantaneous transformation method (also known as the Z transformation; Bartholomew et al., 1981), and dividing the total volume of O_2 consumed/CO_2 produced by the duration of the presence of the hummingbird's head in the mask (Bartholomew and Lighton, 1986). Initially described by Bartholomew et al. (1981), correct application of the Z transformation requires knowing the effective respirometry system volume and assumes that the relative rate at which the changing fractional gas concentration approaches equilibrium is constant. One disadvantage of this approach is that the correction will greatly amplify all changes in fractional gas concentration values, including those due to analyzer noise or drift. Despite its washed out appearance, the total area of the trace of recorded fractional O_2 and CO_2 concentrations below or above, respectively, baseline ambient levels is proportional to the instantaneous metabolic rate of the hummingbird multiplied by the period of time its head was in the mask and expired gases were being captured. Functions for integrating the area of the O_2 or CO_2 trace with respect to baseline values are available in many commercial software packages designed for use with respirometry equipment and from experienced practitioners of respirometry such as Mark Chappell (http://warthog.ucr.edu/). In their application of feeder-mask respirometry on free-ranging Anna's hummingbirds (*C. anna*) Bartholomew and Lighton (1986) noted that the total volume of O_2 consumed during each feeder-mask visitation was linearly related to the feeding sally duration. They showed that dividing the total volume of O_2 consumed during a sally (the entire volume of O_2 'missing' as a result of the feeding event) by sally duration (presence of the bird's head within the feeder-mask) yielded estimates of instantaneous $\dot{V}_{O_2}$ comparable to those calculated using the Z transformation applied

to recordings from a subset of feeding events long enough to result in equilibrium fractional O_2 concentrations being reached (Bartholomew and Lighton, 1986). Feeding sally duration can be accurately determined via video recordings of feeding events taken from angles perpendicular to feeder orientation. Alternatively, sally durations can be measured by detecting the presence of the hummingbird head in the mask as it occludes a photoresistor placed on the underside of the mask (Bartholomew and Lighton, 1986; e.g. Berger and Hart, 1972; Welch et al., 2006; Welch and Suarez, 2008) or a beam of infrared (IR) light between an emitter and detector transecting the mask (e.g. Welch and Suarez, 2007; Welch et al., 2007).

It is important to note that there are more than one type of flow meters acceptable for use with feeder-mask respirometry, but there are important potential limitations to the use of each. Rotameters, which measure volumetric air flow rates based on the competing forces imparted by the air on a float (a small ball typically made of glass or steel) lifted through a vertically oriented, tapered tube and the force of gravity dragging the bore down. The volumetric flow rates reported by such flow meters must be manually and continuously corrected to standard temperature and pressure (STP) and their inherently visual and non-electrical nature makes the tedious to use alongside contemporary gas analyzers and pumps. Mass flow meters often included as part of standard respirometric systems, on the other hand, measure the molar mass, rather than volume, of air flowed through them, with the flow rate typically expressed in units of STP-corrected volume per time. These meters are, by comparison to rotameters, relatively unaffected by ambient temperature and pressure and usually provide digital outputs easily recorded along with the rest of the respirometric data. Many work by measuring the pressure drop of the gas in the airline across a restriction. Alternatively, the commonly available thermal mass flow meter works by heating incoming air and measuring its ability to transfer that heat downstream, which varies as a function of flow rate. Importantly, these flow meters are typically calibrated for use specifically with ambient air or simply nitrogen. Because helium and CO_2 are significantly more thermally conductive than ambient air or nitrogen, most thermal mass flow meters will report artificially high flow rates when helium and CO_2 levels in the airstream rise. Correction factors can typically be obtained from the manufacturer, but their accurate usage requires knowledge of the precise composition of the air mixture. CO_2 levels present in excurrent air from a mask are typically not high enough to warrant concern; the resulting error is typically less than the flow meters specified accuracy. However, when variable gas mixtures (e.g. heliox) are infused into a chamber as hummingbirds hover at a feeder-mask, thermal mass flow meters can report significantly inaccurate flow rates.

3. Case studies: exemplifying the utility of feeder-mask respirometry in hummingbirds: tracking of metabolic substrate use and its relationship to foraging and flight behaviour

In addition to providing insights into how the metabolic cost of hovering flight varies with environmental features such as oxygen availability and air density, studies employing feeder-mask respirometry have shed light on mechanisms by which energetics, morphology, physiology, and behaviour are intimately linked.

Feeder-mask based measurement of hovering and forward flight $\dot{V}_{O_2}$ in multiple age–sex classes of a given hummingbird species appear to relate to slight variations in wing morphology (e.g. Welch and Suarez, 2008; Clark and Dudley, 2009, 2010). Further, experimental modification of morphological features such as tail length has also been linked to modest, yet significant variation in the cost of flight as a function of forward velocity (Clark and Dudley, 2009). This variation in the cost of flight may have implications for foraging behaviour, as it has been suggested that lower costs of flight in less territorially dominant age–sex classes partially offset their reduced

ability to gain and hold the most profitable territories (Welch and Suarez, 2008).

A wealth of information regarding avian energetics has been gained by employing affixed-mask respirometry to track the type of fuels oxidized during flight as inferred from the respiratory quotient or respiratory exchange ratio (RQ or RER; e.g. Tucker, 1968, 1972; Butler et al., 1977; Hudson and Bernstein, 1983; Rothe et al., 1987; Ward et al., 2001, 2002; Bundle et al., 2007). Studies employing feeder-mask respirometry on hummingbirds, often in combination with complimentary techniques such as diet manipulation and isotopic tracking, have provided additional, unique insights into the relationship between metabolic fuels and avian energetics. Such studies have revealed that fasted hummingbirds are capable of fueling energetically expensive hovering flight virtually exclusively via fat oxidation (Suarez et al., 1990; Welch et al., 2006, 2007; Welch and Suarez, 2007). The ability to fuel flight completely, or nearly completely, with fatty acid oxidation is recognized as an ability common to many migratory avian species (reviewed in Weber, 2009). Before it had been empirically demonstrated in birds via application of respirometry, Pearson himself assumed this was true when he calculated whether ruby throated hummingbirds (*Archilochus colubris*) could possibly cross the Gulf of Mexico (a 500+ mile flight over water) during their migratory journey (Pearson, 1950). There are obvious advantages to this ability for long distance aerial migrants such as weight savings as a result of the relatively high density (energy content per unit stored fuel mass) of fat as a stored form of energy, the permitted sparing of body protein reserves, and the ability to sustain performance for long periods without feeding (Weber, 2009). However, many migratory species feed on diets rich in fatty acids and may even benefit from specializing on diets rich in particular unsaturated fatty acids during premigratory foraging periods (Weber, 2009). The specialization of hummingbirds on a diet rich in simple sugars, even during premigratory foraging, means that fat storage results almost exclusively from conversion of ingested sugars, with the net loss of some ingested calories due to the cost of this conversion. Calculating the respiratory quotient during hovering in both fasted and fed hummingbirds, Suarez et al. demonstrated that they possessed the capacity to switch rapidly between fat and carbohydrate as a fuel source (Suarez et al., 1990). This ability appears remarkable, even among birds. Further, using feeder-mask respirometry in combination with stable isotope tracking of exhaled carbon, it has been shown that, once nectar is available during foraging, hummingbirds mobilize newly ingested sugars for use in fueling hovering flight more rapidly, and fuel exercise metabolism more completely from this exogenous energy source, than any other vertebrate thus far examined (Welch et al., 2006, 2008; Welch and Suarez, 2007). For example, Welch et al. (2008) report that, on average, rufous hummingbirds (*S. rufus*) rely on exogenous sugar for 90% of the metabolic fuel oxidized in support of hovering flight after just 15 min of access to a nectar source. Further, they calculated that $94.7 \pm 1.4\%$ ($N = 10$) of rufous hummingbird hovering metabolism is supported by exogenous sugar during the period 30 min to 1 h after gaining access to nectar (Welch et al., 2008). This ability is remarkable considering humans can support only 32.4% of metabolism during moderate intensity exercise (50% of maximum sustained $\dot{V}_{O_2}$) 120–150 min after ingesting a glucose + sucrose solution (Jentjens et al., 2004). Similar data on capacities for the use of exogenous fuels during exercise is not available for a wide range of vertebrates. This is because while coupling of voluntary food consumption and measurement of metabolic rate and determination of fuel nature and source during exercise is possible with a feeder-mask setup, and with cooperative human subjects, it is not easily accomplished with many other animals. Using fat to fuel flight when nectar is available consistently during foraging periods is energetically inefficient because 15% of the energetic content of sugars converted to fat is lost (Suarez et al., 1990). Additionally, if ingested sugars are oxidized

to fuel flight at roughly the same rate they would be converted to energy-dense fat, the weight savings afforded by this conversion is never realized. In this way, hummingbirds maximize the caloric profit of their nectar intake with regular, intermittent foraging during the day, while reserving energy-dense fat stores for use during fasting periods and migratory flights.

It is argued that greater reliance on fatty acid oxidation at low exercise intensity and progressively increased reliance on carbohydrate oxidation at higher aerobic exercise intensity are a highly conserved feature of mammalian physiology (McClelland, 2004). This phenomenon, described as part of the "crossover concept" of fuel use during exercise presented by Brooks and Mercier (Brooks and Mercier, 1994; Brooks, 1997), clearly does not hold true for birds. In the case of fasted and/or migrating birds, the type of fuel used during sustained flight is constrained to available energy stores of sufficient size (namely, fat stores). However, in hummingbirds, the above evidence shows that it is simply the availability of dietary sugars from floral nectar which determines the fuel being used. A similar link between exogenous sugar availability and fuel use during exercise has been discovered for at least one species of nectarivorous bat (also using feeder-mask respirometry; Welch et al., 2008). The shared dietary ecology of glossophagine phyllostomid bats and hummingbirds suggests the importance of foraging strategy and diet type in the evolution of their capacities for fuel use. However, other shared features of their physiology and life history may also be relevant to the evolution of such fuel use capacities including small body size, use of flight, and relatively high metabolic rate.

Application of the feeder-mask respirometry technique has demonstrated that with the capacity to mobilize and rely exclusively upon newly ingested sugars to fuel hovering flight hummingbirds exploit an energetic advantage. Hummingbirds gain the ability to both spare existing fat stores as well as avoid paying the energetic penalties associated with first synthesizing fatty acids from ingested sugars before oxidizing these fuels to support ongoing metabolic demands (Suarez et al., 1990; Welch et al., 2006). Hummingbirds capable of maintaining territorial control over a foraging patch from which they can feed at frequent, regular intervals throughout the day can improve their net energy intake rate by a substantial margin compared to hummingbirds compelled to repeatedly build and subsequently catabolize fat stores throughout the day. These findings from studies employing feeder-mask respirometry demonstrate how hummingbird dietary ecology, behaviour, and physiology are fine tuned to permit maintenance of energy balance. The portability of the feeder-mask respirometry setup and the readiness with which wild hummingbirds will take to using the feeder-mask (e.g. Bartholomew and Lighton, 1986) mean future studies can be undertaken to examine this phenomenon and its relationship to the natural foraging and territorial behaviour of hummingbirds in their native habitats.

While it has long been known that the stoichiometric relationship of ATP production to oxygen consumption varies by 15–19% depending upon whether carbohydrates or fatty acids serve as the metabolic substrate (Brand, 1994, 2005), empirical demonstration of this phenomenon in whole animals during exercise has been lacking. Recently, feeder-mask respirometry has been used to demonstrate the effect of this phenomenon in hovering hummingbirds. In agreement with previous studies on isolated mitochondria, cells, and tissues, the research team showed that hovering hummingbirds consumed 16–18% more oxygen when oxidizing fatty acids than when oxidizing carbohydrate, even as the mechanical costs of hovering remained essentially constant (Fig. 7; Welch et al., 2007). This study validates feeder-mask respirometry as a method for accurately estimating ATP turnover rates (Welch et al., 2007). Further, these findings suggest how fuel type can have implications for behaviour through its effect on oxygen requirements (Welch et al., 2007). The increased oxygen requirements associated with fatty acid oxidation may have important consequences for flight performance when oxygen is limiting,
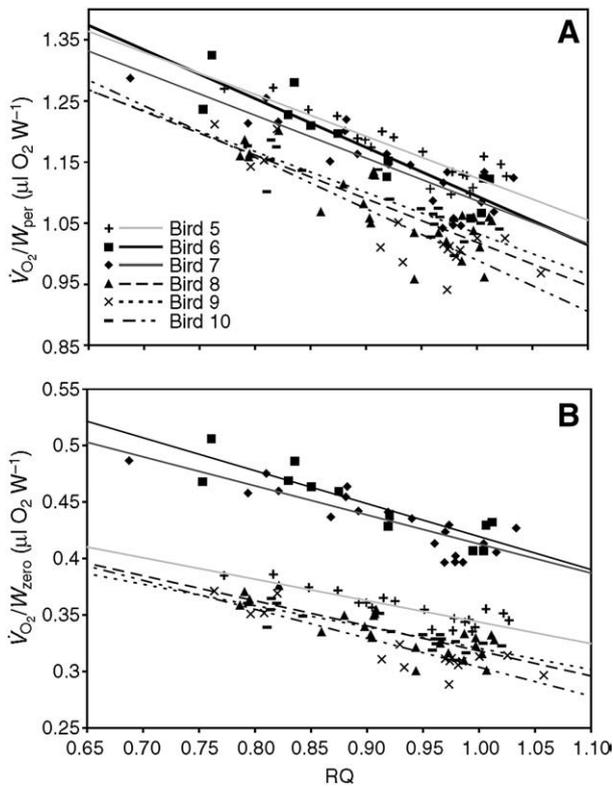
Fig. 7. Oxygen consumption rate per unit hovering power output (V_{O_2}/W) in relation to respiratory quotient (RQ = V_{CO_2}/V_{O_2}) in rufous hummingbirds (*Selasphorus rufus*). Power output was estimated using equations in Ellington (1984). Estimates of V_{O_2}/W are presented assuming either (A) perfect (P_{per}) or (B) zero (P_{zero}) elastic storage of kinetic energy between stroke transitions. Average increases in V_{O_2}/W when hummingbirds oxidize pure fat (predicted V_{O_2}/W when RQ = 0.71) compared to when oxidizing pure carbohydrate (predicted V_{O_2}/W when RQ = 1.0) are 16.8 ± 1.7% when assuming (A) perfect (P_{per}) and 16.2 ± 2.1% when assuming (B) zero elastic storage (P_{zero}). Increases predicted by consensus P/O ratios for fat or carbohydrate oxidation range between 15–18% (Brand, 2005). Figure from Welch et al. (2007).

such as at elevation. Under certain conditions, the ability of hummingbirds to hold nectar-rich territories, and thus continually refuel and rely upon carbohydrate oxidation, may confer competitive performance advantages over hummingbirds lacking such resources and forced to rely more on oxidation of fat stores. Clearly, there are other factors that may be more relevant in determining the outcomes of competitive interactions between hummingbirds, such as morphological features like wing-loading and capacities for burst flight performance (Altshuler, 2006). However, these findings may shed light on patterns of outcomes of competitive encounters in instances when factors like wing-loading and size do not differ significantly, such as interactions between two members of a hummingbird species of the same age and sex, or when these outcomes seem inexplicable except by invocation of the 'resident always wins' hypothesis (e.g. territory holding by 'competitively inferior' classes of rufous hummingbirds; Carpenter et al., 1993). The mechanisms by which fuel type impacts oxygen consumption at a given ATP turnover rate are shared by all animals. Thus, this phenomenon may apply even in those species in which dietary status only affects the fuel mixture in use by degrees, provided restrictions to oxygen availability at the tissue level are a limiting factor in performance.

4. Future directions: new and continuing applications for feeder-mask respirometry in the study of hummingbird flight energetics

While feeder-mask based respirometry applied to the study of hovering and forward flight energetics in hummingbirds will remain a

particularly valuable technique for the foreseeable future, this approach offers additional, immediate opportunities for advancing the study of avian (and, more generally, vertebrate) energetics. The non-invasive nature of mask respirometry means simultaneous or complimentary utilization of other techniques for quantification of metabolic rate during flight, such as the doubly-labelled water technique (DLW; Shaffer, this issue), is possible, permitting cross-validation (Ward et al., 2004). The unique physiology, extreme metabolic rates, and ability of hummingbirds to fly in small arenas, combined with the ease with which feeder-mask respirometry may be applied both in the lab and field, make this system an ideal one in which to explicitly connect an understanding of metabolic, mechanical and aerodynamic performance during flight.

A relatively new technique for estimating energy expenditure, tracking of and injected bolus of ^{13}C-labelled bicarbonate as it mixes with circulation and is respired, offers the advantage of not necessitating sampling during the behaviour of interest (Hambly, 2002; Hambly and Voigt, this issue). The appeal of this technique for the study of uninterrupted, unencumbered bird flight within a wind tunnel or large arena is obvious. However, in each of the studies using this technique for estimation of avian energetics, a curious phenomenon has been observed in which stable carbon isotope ratios in exhaled CO_2 obtained from restrained birds just after a period of activity including flight initially rise, contrary to the expected trend (Hambly, 2002; Hambly et al., 2004a, 2004b, 2004c). The accuracy of this technique is dependent upon assumptions regarding the kinetics of stable carbon isotope ratios in expired CO_2 during the period when the organism transitions to, and from, periods of activity. However, samples are typically unobtainable from most birds during these transitional periods. These unusual isotopic ratio kinetics immediately following the active period suggest that changes in the isotope levels in expired breath occurring over the period of activity may not be straightforward. Unlike in those birds that have thus far been studied, expired CO_2 may readily be sampled from hummingbirds during periods of perching, hovering (Welch et al., 2006; Welch and Suarez, 2007), or forward flight (Clark and Dudley, 2009) using feeder-masks. Further, respirometric measurements of hovering or forward flight metabolic rate may be taken during the same feedings events. Thus, feeder-mask respirometry on hummingbirds may offer a unique tool to investigate the 'black box' of labelled bicarbonate kinetics during periods of flight and can offer a means of validating the accuracy of, or identifying means for correcting, energy estimates using the ^{13}C-labelled bicarbonate technique.

The increasing quality and affordability of high speed video equipment have made the study of hummingbird wing beat kinematics possible for more researchers in more environments. The linking of kinematics and energetics via coupling of feeder-mask respirometry with high speed videography has provided valuable insights into the ways in which biomechanics and aerodynamics relate to energetics (e.g. Wells, 1993; Chai and Dudley, 1995, 1996; Chai et al., 1998; Fernandez and Dudley, 2009). More recently, hummingbird researchers have employed particle image velocimetry as a means of gathering even more data regarding the aerodynamics of hovering flight (Altshuler et al., 2009; Warrick et al., 2005, 2009). However, respirometry and PIV have never been directly coupled in the study of hummingbird flight. Clearly, it is impractical to perform feeder-mask respirometry at the same time as PIV, because of the damaging effects aerosolized olive oil droplets would have on expensive filters, air pumps, and gas analyzers. However, parallel studies, repeated on the same individual hummingbirds, are possible. A sham feeder-mask and air pump fitted with a disposable filter can be employed during PIV studies and then replaced with the working feeder-mask and respirometric equipment under the same environmental conditions after oil droplets have dispersed. In this way, a nearly direct pairing of aerodynamic and metabolic analysis would be possible, to the benefit of an understanding of how efficiently metabolic power input is translated into mechanical and aerodynamic power output.

Acknowledgements

Appendix A. Respirometry equations. All equations presented below are adapted from those listed in Withers (1977) and Lighton (2008)

Key

BP	Barometric pressure
F_eCO_2	Fractional concentration of CO_2 in the excurrent airstream (air leaving the mask)
F_iCO_2	Fractional concentration of CO_2 in the incurrent airstream (air entering the mask; in the case of ambient air, this value is typically around 0.0005)
F_eO_2	Fractional concentration of oxygen in the excurrent airstream (air leaving the mask)
F_iO_2	Fractional concentration of oxygen in the incurrent airstream (air entering the mask; in water and CO_2-free ambient air this is typically 0.2095)
FR	Flow rate of the excurrent air (air leaving the mask; note: depending on the equation used below, this may be the flow rate of air scrubbed of water and CO_2, or scrubbed of water only)
RQ	Respiratory quotient; $= \dot{V}_{CO_2}/\dot{V}_{O_2}$; note: in the equations below in which it appears, RQ is assumed to be estimated because fractional concentrations of either CO_2 or O_2 are not measured
$\dot{V}_{CO_2}$	CO_2 production rate (typically expressed in volume of gas/unit time; e.g. ml CO_2/h)
$\dot{V}_{O_2}$	Oxygen consumption rate (typically expressed in volume of gas/unit time; e.g. ml O_2/h)
WVP	Water vapour pressure

Eq. (1.1). O_2 consumption rate when both CO_2 and water have been scrubbed from the line prior to flow rate measurement (e.g. when there is no subsampling and the pump and flow meter are plumbed directly in line with oxygen analyzer; Fig. 3).

$$\dot{V}_{O_2} = FR(F_iO_2 - F_eO_2) / (1 - F_iO_2) \tag{1.1}$$

Eq. (1.2). CO_2 consumption rate when both CO_2 and water have been scrubbed from the line prior to flow rate measurement (e.g. as described in Eq. (1.1)). The accuracy of this simple equation relies on the assumption that the air incurrent into the mask is entirely free of CO_2. With almost all applications of mask respirometry it is highly unlikely that air entering the mask will be free of CO_2. However, when the air drawn into the mask contains very low levels of CO_2 (e.g. when drawing ambient air with CO_2 concentration $\approx 0.05\%$) the introduced error is negligible when compared to errors in flow rate measurement. Given this, Eq. (1.2) is acceptably accurate for use in determination of CO_2 consumption rate and is much less cumbersome than the technically more correct Eq. (1.3) (see below).

$$\dot{V}_{CO_2} = FR(F_eCO_2 - F_iCO_2) \tag{1.2}$$

Eq. (1.3). CO_2 production rate when both CO_2 and water have been scrubbed from the line prior to flow rate measurement (e.g. as described in Eqs. (1.1) and (1.2)). This equation is preferred over Eq. (1.2) when the CO_2 concentration (including CO_2 introduced by the experimental subject) in the excurrent air from the mask exceeds 0.5%, as the error such CO_2 levels introduce when using Eq. (1.2) are significant.

$$\dot{V}_{CO_2} = FR(F_eCO_2 - F_iCO_2) / (1 - F_eCO_2 + F_iCO_2 / RQ) \tag{1.3}$$

(when $\dot{V}_{O_2}$ is *not* independently measured)or

$$\dot{V}_{CO_2} = \left[FR(F_eCO_2 - F_iCO_2) - F_eCO_2\left(V_{O_2}\right) \right] / (1 - F_eCO_2)$$

(when $\dot{V}_{O_2}$ is independently measured).

Eq. (1.4). Method for calculating flow rate of water vapour-free, 'dry' air when chemical scrubbing of water is *not* performed prior to flow rate measurement (e.g. when the flow rate is so high as to make chemical scrubbing of the airstream impractical). The calculated flow rate is for use in Eqs. (1.5) and (1.6) below.

$$FR = FR_u(BP - WVP) / BP \tag{1.4}$$

Eq. (1.5). Oxygen consumption rate when water is scrubbed (or the flow rate has been corrected using Eq. (1.4)) from the airstream prior to flow rate measurement but CO_2 is *not* scrubbed from the airstream at any point. Note: this is likely in a mask respirometry setup where a relatively high flow rate through the mask is used (>2 L/min), and the excurrent airstream is subsampled for analysis. In this case, it is nonetheless wise to scrub the subsampled airstream of water upstream of the oxygen or CO_2 from the analyzers.

$$\dot{V}_{O_2} = FR(F_iO_2 - F_eO_2) / [1 - F_iO_2(1 - RQ)] \tag{1.5}$$

(when $\dot{V}_{CO_2}$ is *not* independently measured) or

$$\dot{V}_{O_2} = FR[(F_iO_2 - F_eO_2) - F_iO_2(F_eCO_2 - F_iCO_2)] / (1 - F_iO_2)$$

(when $\dot{V}_{\backslash CO_2}$ is independently measured).

Eq. (1.6). CO_2 production rate when water has been scrubbed (or the flow rate has been corrected using Eq. (1.4)) from the airstream prior to flow rate measurement but CO_2 is *not* scrubbed from the airstream at any point.

$$\dot{V}_{CO_2} = FR(F_eCO_2 - F_iCO_2) / \{1 - F_iCO_2[1 - (1 / RQ)]\} \tag{1.6}$$

(when $\dot{V}_{O_2}$ is *not* independently measured) or

$$\dot{V}_{CO_2} = FR[(F_eCO_2 - F_iCO_2) + F_iCO_2(F_iO_2 - F_eO_2)] / (1 + F_iCO_2)$$

(when $\dot{V}_{O_2}$ is independently measured).

References

Altshuler, D.L., 2006. Flight performance and competitive displacement of hummingbirds across elevational gradients. Am. Nat. 167, 216–229.

Altshuler, D.L., Chai, P., Chen, J.S.P., 2001. Hovering performance of hummingbirds in hyperoxic gas mixtures. J. Exp. Biol. 204, 2021–2027.

Altshuler, D.L., Dudley, R., McGuire, J.A., 2004. Resolution of a paradox: hummingbird flight at high elevation does not come without a cost. Proc. Natl Acad. Sci. U. S. A. 101, 17731–17736.

Altshuler, D.L., Princevac, M., Hansheng, P., Lozano, J., 2009. Wake patterns of the wings and tail of hovering hummingbirds. Exp. Fluids 46, 835–846.

Bartholomew, G.A., Lighton, J.R.B., 1986. Oxygen consumption during hover-feeding in free-ranging Anna hummingbirds. J. Exp. Biol. 123, 191–199.

Bartholomew, G.A., Vleck, D., Vleck, C.M., 1981. Instantaneous measurements of oxygen consumption during pre-flight warm-up and post-flight cooling in Sphingid and Saturniid moths. J. Exp. Biol. 90, 17–32.

Benedict, F.G., Fox, E.L., 1927. The gaseous metabolism of large wild birds under aviary life. Proc. Am. Philos. Soc. 66, 511–534.

Berger, M., 1974. Energiewechsel von kolibris beim schwirrflug unter höhenbedingungen. J. Ornithol. 115, 273–288.

Berger, M., Hart, J.S., 1972. Die atmung beim kolibri *Amazilia fimbriata* während des schwirrfluges bei verschiedenen umgebungstemperaturen. J. Comp. Physiol. A 81, 363–380.

Bernstein, M.H., 1976. Ventilation and respiratory evaporation in the flying crow, *Corvus ossifragus*. Respir. Physiol. 26, 371–382.

Bernstein, M.H., Thomas, S.P., Schmidt-Nielsen, K., 1973. Power input during flight of the fish crow, *Corvus Ossifragus*. J. Exp. Biol. 58, 401–410.

Brand, M.D., 1994. The stoichiometry of proton pumping, ATP synthesis and oxygen consumption in mitochondria. Biochemistry 16, 20–24.

Brand, M.D., 2005. The efficiency and plasticity of mitochondrial energy transduction. Trans. Biochem. Soc. 33, 897–903.

Brooks, G.A., 1997. Importance of the 'crossover' concept in exercise metabolism. Clin. Exp. Pharmacol. Physiol. 24, 889–895.

Brooks, G.A., Mercier, J., 1994. Balance of carbohydrate and lipid utilization during exercise: the "crossover" concept. J. Appl. Physiol. 76, 2253–2261.

Bundle, M.W., Hansen, K.S., Dial, K.P., 2007. Does the metabolic rate-flight speed relationship vary among geometrically similar birds of different mass? J. Exp. Biol. 210, 1075–1083.

Butler, P.J., 2000. Heart rate and rate of oxygen consumption during flight of the barnacle goose, *Branta leucopsis*. Comp. Biochem. Physiol. A 126, 379–385.

Butler, P.J., West, N.H., Jones, D.R., 1977. Respiratory and cardiovascular responses of the pigeon to sustained, level flight in a wind-tunnel. J. Exp. Biol. 71, 7–26.

Carpenter, F.L., Hixon, M.A., Temeles, E.J., Russell, R.W., Paton, D.C., 1993. Exploitative compensation by subordinate age–sex classes of migrant rufous hummingbirds. Behav. Ecol. Sociobiol. 33, 305–312.

Chai, P., 1997. Hummingbird hovering energetics during moult of primary flight feathers. J. Exp. Biol. 200, 1527–1536.

Chai, P., Dudley, R., 1995. Limits to vertebrate locomotor energetics suggested by hummingbirds hovering in heliox. Nature 377, 722–725.

Chai, P., Dudley, R., 1996. Limits to flight energetics of hummingbirds hovering in hypodense and hypoxic gas mixtures. J. Exp. Biol. 199, 2285–2295.

Chai, P., Harrykissoon, R., Dudley, R., 1996. Hummingbird hovering performance in hyperoxic heliox: effects of body mass and sex. J. Exp. Biol. 199, 2745–2755.

Chai, P., Chang, A.C., Dudley, R., 1998. Flight thermogenesis and energy conservation in hovering hummingbirds. J. Exp. Biol. 201, 963–968.

Clark, C.J., Dudley, R., 2009. Flight costs of long, sexually selected tails in hummingbirds. Proc. R. Soc. Lond. B Biol. Sci. 276, 2109–2115.

Clark, C.J., Dudley, R., 2010. Hovering and forward flight energetics in Anna's and Allen's hummingbirds. Physiol. Biochem. Zool. 83, 654–662.

Ellington, C.P., 1984. The aerodynamics of hovering insect flight. VI. Lift and power requirements. Proc. R. Soc. Lond. B Biol. Sci. 305, 145–181.

Epting, R.J., 1980. Functional dependence of the power for hovering on wing disc loading in the hummingbirds. Physiol. Zool. 53, 347–357.

Evangelista, D., Fernández, M.J., Berns, M.S., Hoover, A., Dudley, R., 2010. Hovering energetics and thermal balance in Anna's hummingbirds (*Calypte anna*). Physiol. Biochem. Zool. 83, 406–413.

Fernandez, M.J., Dudley, R., 2009. Elevational variation in flight mechanics and energetics of the giant Andean hummingbird. Integr. Comp. Biol. 49, E54.

Gessaman, J.A., 1980. An evaluation of heart rate as an indirect measure of daily energy metabolism of the American kestrel. Comp. Biochem. Physiol. A 65, 273–289.

Halsey, L.G., Shepard, E.L.C., Quintana, F., Gomez Laich, A., Green, J.A., Wilson, R.P., 2009. The relationship between oxygen consumption and body acceleration in a range of species. Comp. Biochem. Physiol. A 152, 197–202.

Hambly, C., 2002. Cost of flight in the zebra finch (*Taenopygia guttata*): a novel approach based on elimination of ^{13}C labelled bicarbonate. Comp. Physiol. B 172, 529–539.

Hambly C., Voigt C.C., this issue. Measuring energy expenditure in birds using bolus injections of (13)C labelled Na-bicarbonate. Comp. Biochem. Physiol. A Mol. Integr. Physiol. (2010 May 24, Electronic publication ahead of print).

Hambly, C., Harper, E.J., Speakman, J.R., 2004a. The energy cost of loaded flight is substantially lower than expected due to alterations in flight kinematics. J. Exp. Biol. 207, 3969–3976.

Hambly, C., Harper, E.J., Speakman, J.R., 2004b. The energetic cost of variations in wing span and wing asymmetry in the zebra finch *Taeniopygia guttata*. J. Exp. Biol. 207, 3977–3984.

Hambly, C., Pinshow, B., Wiersma, P., Verhulst, S., Piertney, S.B., Harper, E.J., Speakman, J.R., 2004c. Comparison of the cost of short flights in a nectarivorous and a non-nectarivorous bird. J. Exp. Biol. 207, 3959–3968.

Hudson, D.M., Bernstein, M.H., 1981. Hovering energetics and thermal balance in Anna's hummingbirds (*Calypte anna*). J. Exp. Biol. 90, 267–281.

Hudson, D.M., Bernstein, M.H., 1983. Gas exchange and energy cost of flight in the white-necked raven, *Corvus cryptoleucus*. J. Exp. Biol. 103, 121–130.

Jentjens, R.L.P.G., Venables, M.C., Jeukendrup, A.E., 2004. Oxidation of exogenous glucose, sucrose, and maltose during prolonged cycling exercise. J. Appl. Physiol. 96, 1285–1291.

Kleiber, M., 1975. The Fire of Life: An Introduction to Animal Energetics, 2nd ed. Robert E. Kreiger Publishing Company, Malabar, Florida.

Lasiewski, R.C., 1963. Oxygen consumption of torpid, resting, active, and flying hummingbirds. Physiol. Zool. 36, 122–140.

Lasiewski, R.C., Dawson, W.R., 1967. A re-examination of the relation between standard metabolic rate and body weight in birds. Condor 69, 13–23.

Lavoisier, A., 1777. Experiences sur la respiration des animaux — et sur les chanements qui arrivent a l'air en passant par leur poumon. Memoire lu a l'Academie des sciences. French Academy of Science, France, p. 185.

Lighton, J.R.B., 2008. Measuring Metabolic Rates: A Manual for Scientists. Oxford University Press, New York.

McClelland, G.B., 2004. Fat to the fire: the regulation of lipid oxidation with exercise and environmental stress. Comp. Biochem. Physiol. B 139, 443–460.

McNab, B.K., 2006. The relationship among flow rate, chamber volume and calculated rate of metabolism in vertebrate respirometry. Comp. Biochem. Physiol. A 145, 287–294.

Pearson, O.P., 1950. The metabolism of hummingbirds. Condor 52, 145–152.

Rayner, J.M.V., 1994. Aerodynamic corrections for the flight of birds and bats in wind tunnels. J. Zool. 234, 537–563.

Rayner, J.M.V., 1999. Estimating power curves of flying vertebrates. J. Exp. Biol. 202, 3449–3461.

Rothe, H., Biesel, W., Nachtigall, W., 1987. Pigeon flight in a wind tunnel. II. Gas exchange and power requirements. J. Comp. Physiol. B 157, 99–109.

Schuchmann, K., 1979. Metabolism of flying hummingbirds. Ibis 121, 85–86.

Shaffer S.A., this issue. A review of seabird energetics using the doubly labeled water method. Comp. Biochem. Physiol. A Mol. Integr. Physiol. PMID: 20656049 (2010 Jul 22, Electronic publication ahead of print).

Stiles, F.G., 1971. Time, energy, and territoriality of the Anna hummingbird (*Calypte anna*). Sci. New Ser. 173, 818–821.

Suarez, R.K., 1992. Hummingbird flight: sustaining the highest mass-specific metabolic rates among vertebrates. Experientia 48, 565–570.

Suarez, R.K., Brown, G.S., Hochachka, P.W., 1986. Metabolic sources of energy for hummingbird flight. Am. J. Physiol. Regul. Integr. Comp. Physiol. 251, R537–R542.

Suarez, R.K., Lighton, J.R.B., Moyes, C.D., Brown, G.S., Gass, C.L., Hockachka, P.W., 1990. Fuel selection in rufous hummingbirds: ecological implications of metabolic biochemistry. Proc. Natl Acad. Sci. U. S. A. 87, 9207–9210.

Tobalske, B.W., 2009. Effects of flight speed upon muscle activity and metabolic rate in hummingbirds. Comp. Biochem. Physiol. A 153, S122.

Torre-Bueno, J., Larochelle, J., 1978. The metabolic cost of flight in unrestrained birds. J. Exp. Biol. 75, 223–229.

Tucker, V.A., 1966. Oxygen consumption of a flying bird. Science 154, 150–151.

Tucker, V.A., 1968. Respiratory exchange and evaporative water loss in the flying budgerigar. J. Exp. Biol. 48, 67–87.

Tucker, V.A., 1972. Metabolism during flight in the laughing gull, *Larus atricilla*. Am. J. Physiol. 222, 237–245.

Ward, S., Moller, U., Rayner, J.M.V., Jackson, D.M., Bilo, D., Nachtigall, W., Speakman, J.R., 2001. Metabolic power, mechanical power and efficiency during wind tunnel flight by the European starling *Sturnus vulgaris*. J. Exp. Biol. 204, 3311–3322.

Ward, S., Bishop, C.M., Woakes, A.J., Butler, P.J., 2002. Heart rate and the rate of oxygen consumption of flying and walking barnacle geese (*Branta leucopsis*) and bar-headed geese (*Anser indicus*). J. Exp. Biol. 205, 3347–3356.

Ward, S., Moller, U., Rayner, J.M.V., Jackson, D.M., Nachtigall, W., Speakman, J.R., 2004. Metabolic power of European starlings *Sturnus vulgaris* during flight in a wind tunnel, estimated from heat transfer modelling, doubly labelled water and mask respirometry. J. Exp. Biol. 207, 4291–4298.

Warrick, D.R., Tobalske, B.W., Powers, D.R., 2005. Aerodynamics of the hovering hummingbird. Nature 435, 1094–1097.

Warrick, D.R., Tobalske, B.W., Powers, D.R., 2009. Lift production in the hovering hummingbird. Proc. R. Soc. Lond. B Biol. Sci. 276, 3747–3752.

Weber, J., 2009. The physiology of long-distance migration: extending the limits of endurance metabolism. J. Exp. Biol. 212, 593–597.

Welch Jr., K.C., Suarez, R.K., 2007. Oxidation rate and turnover of ingested sugar in hovering Anna's (*Calypte anna*) and rufous (*Selasphorus rufus*) hummingbirds. J. Exp. Biol. 210, 2154–2162.

Welch Jr., K.C., Suarez, R.K., 2008. Altitude and temperature effects on the energetic cost of hover-feeding in migratory rufous hummingbirds, *Selasphorus rufus*. Can. J. Zool. 86, 161–169.

Welch Jr., K.C., Bakken, B.H., Martínez del Rio, C., Suarez, R.K., 2006. Hummingbirds fuel hovering flight with newly ingested sugar. Physiol. Biochem. Zool. 79, 1082–1087.

Welch Jr., K.C., Altshuler, D.L., Suarez, R.K., 2007. Oxygen consumption rates in hovering hummingbirds reflect substrate-dependent differences in P/O ratios: carbohydrate as a 'premium fuel'. J. Exp. Biol. 210, 2146–2153.

Welch Jr., K.C., Herrera M., L.G., Suarez, R.K., 2008. Dietary sugar as a direct fuel for flight in the nectarivorous bat *Glossophaga soricina*. J. Exp. Biol. 211, 310–316.

Wells, D.J., 1993. Muscle performance in hovering hummingbirds. J. Exp. Biol. 178, 39–57.

Withers, P.C., 1977. Measurement of VO_2, VCO_2, and evaporative water loss with a flow-through mask. J. Appl. Physiol. 42, 120–123.

Woakes, A.J., Butler, P.J., 1983. Swimming and diving in tufted ducks, *Aythya fuligula*, with particular reference to heart rate and gas exchange. J. Exp. Biol. 107, 311–329.

Wolf, L.L., Hainsworth, F.R., 1971. Time and energy budgets of territorial hummingbirds. Ecology 52, 980.