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Flight Performance and Comparative Energetics of the Giant Andean Hummingbird (Patagona gigas)

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Flight Performance and Comparative Energetics of the Giant Andean Hummingbird (*Patagona gigas*)

By

Maria Jose Fernandez

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University of California, Berkeley

Committee in charge:

Professor Robert Dudley, Chair
Associate Professor Jimmy A. McGuire
Professor Steven Beissinger

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Abstract

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Among vertebrates, hummingbirds (family Trochilidae) represent an extreme outcome in vertebrate physiological design, and are unique in their capacity for sustained hovering flight. Because hovering flight is one of the most energetically demanding forms of locomotion requiring high levels of metabolic power input and mechanical power output, hummingbirds offer a useful system to study their energetics and flight mechanics. There are about 330 species of hummingbirds, of which 70% of the species weigh less than six grams and about 30% are between six and ten grams. Surprisingly, just one species, *Patagona gigas* (the Giant Hummingbird), weighs on average 20 g, making it an outlier in the hummingbird body size distribution.

Because power requirements increase with (body mass)$^{1.17}$, whereas maximum aerobic capacity of volant animals scales negatively with body mass, large body size presents a dual mechanical/metabolic challenge to hovering flight in the Giant Hummingbird. In spite of the challenges of supporting its large body weight, *P. gigas* inhabits a broad altitudinal range, from sea level to 4500 m.a.s.l. At higher altitudes, where the combination of low air density, low partial pressure of oxygen and low temperatures makes both lift generation and flight energetics especially costly to achieve, *P. gigas,* on account of its large body size, is likely to face more extreme energetic restrictions.

The overarching goal of my research program has been to determine the specific physical and environmental constraints that limit upper body size in hummingbirds, and to elucidate those aspects of the Giant Hummingbird lifestyle, physiology, biochemistry and biomechanics that have liberated it from these constraints. First, through measurements of daily, basal, and hovering rates of oxygen consumption in the Giant Hummingbird, I have shown that this species, although a clear outlier in terms of body size, does not deviate significantly from metabolic relationships derived from smaller hummingbirds. During this research I also measured enzyme flux capacities (V_{max} values) of key enzymes in pathways of glucose and fatty acid oxidation in the flight muscles of the Giant Hummingbird as well as smaller species. Because flight muscles account for most of the VO_2 during hovering flight, I was able to accurately estimate metabolic flux rates from respirometric data obtained during hovering flight. My results reveal that hummingbirds share a highly conserved set of pathways for glucose and fatty acid oxidation. In addition, there was a lack of quantitative, mass-dependent interspecific variation in V_{max} values

for most of the enzymes involved in glucose and fat oxidation, in spite of the mass-dependent interspecific variation in hovering metabolic rate found in my first study. These results suggest a "one size fits all" hypothesis, i.e., qualitative as well as quantitative evolutionary conservation of pathways of energy metabolism. The lack of correlation between V_{max} values and flux rates at most steps in energy metabolism, suggests that the interspecific variation in flux through pathways of glucose and fatty acid oxidation during hovering is achieved through modulation of enzyme activities, rather than adjustments in enzyme concentration.

Overall, the allometric analyses and the enzyme flux capacities indicate that the Giant Hummingbird is just a "big" hummingbird and not an allometric outlier: estimates of metabolic parameters fall close to the allometric projections from smaller hummingbirds. However, my previous experiments were done at low elevations where the energetic requirements are lower. Consequently, the occurrence of *P. gigas* at a wide range of altitudes provides an excellent "natural experiment," with which to assess the behavioural, biomechanical and energetic responses of this bird to natural hypobaric variation. Accordingly, I explored the mechanisms used by this species to cope with the enhanced energetic and aerodynamic demands of living at high elevations. I measured flight kinematics (wingbeat frequency and stroke amplitude) and energetics (oxygen consumption) during hovering flight at two elevations spanning a 3700 m elevational gradient along the Andes Mountains of Peru. Contrary to my predictions, the Giant Hummingbird increased wing stroke amplitudes and wingbeat frequencies equally, and thus increased mechanical power output at high elevations relative to sea level. Moreover, oxygen consumption during hovering increased significantly (~33%) at the high elevation site. However, based on the observed increase in mechanical power, the 33% increase in metabolic power was larger than expected. It is unclear why metabolic costs would increase at a substantially greater rate than mechanical power output. Perhaps, exploring how the Giant Hummingbird modulates detailed wingbeat kinematics (e.g. wing rotation, angle of attack, torsion along the wing) might help to explain the unexpected lack of correlation between mechanical and metabolic power. Also, *Patagona gigas gigas* is a subspecies that inhabits mainly low elevations up to 2500 m. Studying this group in comparison to *P. gigas peruviana* (high elevation subspecies) could elucidate the mechanical and physiological mechanisms that have enabled the Giant Hummingbird to adapt to extremely different environmental conditions in spite of the high cost. Moreover, these comparisons may shed some light on the evolutionary trade-offs and constraints that have shaped the history and the evolutionary potential of hummingbird species.

In summary, my dissertation shows that in spite of the large size of the Giant Hummingbird, it is not an outlier in terms of energetics or flight performance when compared to smaller hummingbirds. These findings may suggest that hummingbirds body size distribution and upper body size limit might have an ecological explanation rather than a physiological, biochemical or biomechanical constraint.

Dedico este trabajo a mis hemanos, Fernando y Felipe, por su apoyo incondicional.

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Chapter 1

Comparative Energetics of the Giant Hummingbird (*Patagona gigas*)

Abstract

Hummingbirds (family Trochilidae) represent an extreme outcome in vertebrate physiological design, and are the only birds capable of sustained hovering. The Giant Hummingbird (*Patagona gigas*) is the largest trochilid with a mass of ~20 g, and is found over an altitudinal range from 0 to 4500 m.a.s.l. We report here measurements of daily, basal, and hovering rates of oxygen consumption in the Giant Hummingbird, compare these values with data from other hummingbirds, and assess overall metabolic and allometric limits to trochilid body size. The sustained metabolic scope (i.e., the ratio of daily energy expenditure to basal metabolic rate) in the Giant Hummingbird is higher than that of smaller hummingbirds, but lies below a proposed theoretical maximum value for endotherms. Scaling exponents in the allometric relationships for different modes of energetic expenditure were comparable, suggesting that the Giant Hummingbird, although a clear outlier in terms of body size, does not obviously deviate from metabolic relationships derived from other trochilid taxa.

Introduction

Understanding those factors that impose ceilings on metabolic rate is essential for understanding design constraints on animal physiological performance (Peterson et al. 1990; Hammond and Diamond 1997; Bacigalupe and Bozinovic 2002). These limits may be also reflected in locomotor abilities, behavioural allocation, and energy budgets that ultimately impinge on survivorship (e.g., Weiner 1992; Hammond and Diamond 1997; Speakman 2000). Hummingbirds, because of their ability to sustain hovering flight and apparently extreme physiological performance, represent an outstanding group with which to study limits to mass-specific metabolic rates in vertebrates (Tracy 1977; Suarez 1992; McNab 2002). The wide range of body size in this group (~2 – 20 g) also facilitates allometric characterization of the limits to animal flight performance (Altshuler and Dudley 2002).

Although most of the more than 330 species of hummingbirds weigh less than six grams, with the remainder weighing between 6 – 12 g, there is one puzzling outlier, namely the Giant Hummingbird (*Patagona gigas*). This species typically weighs ~20 g, almost twice the mass of the next largest hummingbird (Dunning 1993; Cotton 1996; Dickinson 2003). In spite of the challenges of supporting its large body weight, *P. gigas* inhabits a broad altitudinal range (from 0 to 4500 m.a.s.l.) along the Andes, from southern Colombia to Argentina and Chile (see Ortiz-Crespo 1974). Like all other hummingbirds, this very large species is an obligate nectarivore and routinely engages in hovering flight. However, flight energetics in this over-sized species are currently unstudied, only limited data on basal metabolic rates for *P. gigas* are available (Lasiewski et al. 1967).

In this study, we characterise rates of energetic expenditure in *P. gigas* and three sympatric trochilid species under a range of activity levels and environmental conditions. Basal metabolic rate (BMR) represents the minimum energetic cost of physiological maintenance under thermoneutral conditions. Hovering metabolic rate (HMR) refers to energetic expenditure during the behaviour of hover-feeding, but does not necessarily correspond to the maximum metabolic rate attainable during flight (see Chai and Dudley 1995; Clark and Dudley 2009). Daily energy expenditure (DEE; also referred to as field metabolic rate when measured on free-ranging animals) measures the rate of energy metabolism of an active organism. DEE includes the BMR and any supplemental costs of thermoregulation, locomotion, foraging, digestion, growth, and reproduction, as well as of other energetic expenditures that yield heat (Nagy 1987).

The scaling of BMR, DEE, and maximum metabolic rates can be highly variable among endotherms (e.g., McNab 1980; Taylor 1982; Ellis 1984; Koteja 1987; Dawson and Olson 1988; Bozinovic 1992; McNab 2002). Previous studies have suggested that high metabolic scopes (i.e., DEE/BMR, meaning that the range through which the aerobic metabolic rate can vary exceeds a factor of seven) are physiologically unsustainable and detrimental to vertebrate survival (Drent and Daan 1980; Peterson et al. 1990; Hammond and Diamond 1997). Given the overriding influence of body mass on rates of energetic expenditure (McNab 2002), we test here the hypothesis that larger hummingbirds are relatively closer to maximum values for sustained metabolic scope. Therefore, we expect to find a stronger scaling relationship between DEE and body mass than for BMR. We compare different levels of energetic expenditure in *P. gigas* with those for other hummingbirds by deriving allometric relationships for BMR, DEE, and HMR using data from this study and previously published information from other trochilid species. Possible allometric limitations on the maximum size of hummingbirds are then indicated by differential scaling of these rates and disproportionate rates of energetic expenditure above a certain body mass.

Material and Methods

We captured four hummingbird species in Chile using mist nets. We caught seven individuals of *Sephanoides sephaniodes* and four *Patagona gigas* in the Andean foothills at San Carlos de Apoquindo (33º 23' S, 70º 31' W), approximately 20 km east of Santiago (~1100 m.a.s.l.). We caught six individuals of *Rhodopis vesper,* five *Oreotrochilus estella* and two *P. gigas* in Chusmiza (19º 40' S, 69º 10' W, ~3580 m.a.s.l.). *Patagona gigas* is sexually monomorphic, and the metabolic data reported here refers to pooled values for individuals of unknown gender. The other three aforementioned species are sexually dimorphic, but given our focus on interspecific differences among hummingbirds, we calculated average metabolic data for different individuals within each species.

We transported hummingbirds to the Pontificia Universidad Católica de Chile in Santiago (33º 27' S, 70º 40' W, 520 m.a.s.l.) where we made most of the metabolic measurements (i.e., of BMR, and HMR). In addition, we measured DEE of *P. gigas* alone at the Estación de Investigaciones Ecológicas Mediterráneas (EDIEM) of the Pontificia Universidad Católica de Chile in San Carlos de Apoquindo, Santiago (33º 23' S, 70º 31' W, 1100 m.a.s.l.). Hummingbirds were housed individually in large fabric cages (90 x 90 x 90 cm) at 25 ºC under a 12:12 light:dark cycle. We provided an artificial nectar solution (13% (weight/volume); Nektar-plus,

Pforzheim, Germany) supplemented with 5% weight/volume of sucrose *ad libitum,* as well as live fruit flies (*Drosophila melanogaster*) as a nutritional supplement.

Daily energy expenditure

We measured DEE in four individual *P. gigas* conducted in an outdoor aviary (6 x 2 x 2 m) during December and January 2002 (corresponding to the austral summer season). The aviary contained nectar feeders (0.75 M sucrose solution), suspended branches for perching, and a water bath; we also provided live fruit flies (*Drosophila melanogaster*). We studied birds in isolation to preclude social interactions. We measured DEE using the doubly-labeled water (DLW) method (Nagy 1980; Nagy and Costa 1980). We used a water mixture enriched with ^{2}H and ^{18}O as provided by the Center for Isotope Research (CIO) at the University of Groningen, The Netherlands. Following the protocol established by the CIO and Bozinovic et al. (2003), we first injected 0.2 ml of DLW into a thigh muscle. After 1 h equilibration, we obtained an initial blood sample (40 μl) collected by toe clipping (Powers and Conley 1994) and then released the bird into the aviary. We flame-sealed blood aliquots and stored at 5 ºC for subsequent analysis. After 24 h, we recaptured the hummingbird and took a second blood sample. We weighed birds using an electronic balance (Sartorius, ± 0.01 g) before and after we took blood samples to determine a mean mass value. We recorded time of sampling to the nearest minute. We sent blood samples to the CIO for analysis of isotope concentrations by isotope ratio mass spectrometry, which we used in turn to estimate rates of CO_2 production as averaged over 24 h. We calculated energy equivalents of these rates assuming metabolism of pure carbohydrate diet (i.e., 21.1 J/ml CO_2; Bozinovic et al., 2005).

Basal metabolic rate

We measured BMR using an open-flow respirometry system in six *P. gigas*, five *O. estella,* seven *S. sephaniodes*, and six *R. vesper*. We measured rates of oxygen consumption on birds resting within a small dark metabolic chamber (630 ml) under postabsorptive conditions at 30 ± 0.5 °C within the appropriate thermoneutral zone for each species (Lasiewski et al. 1967; Bozinovic and Fernández, unpublished data). Measurements were performed at night on non-reproductive individuals. The airflow rate through the metabolic chamber was 550 ml min^{-1}, as monitored by a mass flow controller (Sierra Instruments, Monterey, CA), to ensure adequate gas mixing in the chamber. Before and after air passed through the chamber, Baralyme (Keomed, St. Paul, MN) and Drierite (Hammond, Xenia, OH) removed carbon dioxide and water vapour, respectively. An Applied Electrochemistry O_2 Analyzer (Model S-3A/I, Ametek, Pittsburgh, PA) sampled air every 5 s. We used equations in Withers (1977) to calculate rates of oxygen consumption. An electronic balance (Sartorius, ± 0.01 g) recorded body mass prior to each metabolic measurement. We monitored BMR over 75 min on average (range 60 – 90 min); we accepted as BMR the lowest steady-state value found among all consecutive 5 min measurement intervals.

Hovering metabolic rate

Following the protocols of Bartholomew and Lighton (1986), an open respirometry system was used to measured rates of oxygen consumption during hover-feeding (HMR) of four *P. gigas*, two *O. estella,* five *S. sephaniodes*, and four *R. vesper*. We sampled expired narial gases using a free-standing plastic hummingbird feeder altered to function as a respirometry mask. We used the same method described above to scrub water vapour and CO_2 from the airstream with flow rate maintained at 1000 ml/min. Oxygen concentration of the incoming air was measured with a FOXBOX Oxygen Analyzer (Sable Systems International). When the hovering bird inserted its head into the mask to feed, oxygen depletion in the sampled respiratory flow corresponded to the amount of oxygen consumed by the bird over the duration of the feeding bout (see Bartholomew and Lighton 1986). We estimated hovering metabolic rate from the area under the curve of oxygen concentration versus time, divided by the total duration that the bird's head was inserted in the mask, as derived from the sustained reduction of the oxygen trace (average duration of 6 s, range 3 – 12 s). We recorded five hovering bouts per individual in order to obtain an average value of the rate of oxygen consumption. We measured body mass before each trial using an electronic balance (Sartorius, ± 0.01 g); we used this value as the representative value for subsequent mass-specific estimates of metabolic rate.

Comparative analyses

In addition to obtaining the above measurements, we compiled additional data from the literature for DEE, BMR, and HMR of 13 additional species (DEE: *N*=5 species, Powers and Nagy 1988; Powers and Coley 1994; Weathers and Stiles 1989; BMR: *N*=7 species, Lasiewski 1963; HMR: *N*=9 species, Lasiewski 1963; Wolf and Hainsworth 1971; Berger and Hart 1972; Berger 1974; Epting 1980; Bartholomew and Lighton 1986; Suarez et al. 1990; Chai and Dudley 1996). The combined data were used to estimate scaling relationships for the metabolic rates of hummingbirds under different conditions of energetic expenditure. In addition to analysing regressions based on species means alone, we also incorporated effects of phylogenetic similarity among study taxa by calculating standardised independent contrasts (Felsenstein 1985). We used the complete phylogenetic hypothesis of McGuire et al. (2007), which includes 73 of the approximately 104 currently recognised trochilid genera. We obtained branch lengths from McGuire (pers. comm.), and entered them in Mesquite (Maddison and Maddison 2006) for use in further analyses. Independent contrasts were calculated using the PDAP module in Mesquite (Garland et al. 1993; Midford et al. 2005); all associated regressions were constrained to go through the origin (Garland et al. 1992). Two species for which published metabolic data are available (*Lampornis clemenciae* and *Selasphorus sasin*) were not represented in the phylogeny of McGuire et al. (2007), and these data were accordingly not used in analyses using independent contrasts. To test whether the contrasts were adequately standardised, we regressed the absolute values of the standardized contrast against the standard deviations of the variables (log BMR, log DEE, log HMR and log body mass) (Diaz-Uriarte and Garland 1998). All variables showed no relationship (least square regressions, $P > 0.05$), which indicates that the contrasts are adequately standardised.

Allometric variation in energy expenditure (i.e., in DEE, BMR, and HMR) for hummingbirds, including *P. gigas*, was assessed using least squares linear regressions of log-

transformed mean values for each species versus log-transformed body mass. Each regression was performed twice, once including and once excluding values for *P. gigas*. To test whether elevated rates of energy expenditure scale similarly or differently from BMR, we compared the corresponding regression slopes using an *F* test for homogeneity of slopes (Statistica 5.0). All other statistical analyses were performed using JMP 5.0 (SAS Institute, Cary, NC) and Matlab R2008b (MathWorks Inc., Natick, MA).

Results

Overall, values of BMR, DEE, and HMR differed significantly in *P. gigas* (one-way ANOVA: $F_{2,24} = 130.53$, $P < 0.0001$; Table 1.1 and Fig. 1.1). DEE differed significantly from HMR (all-pairs Tukey HSD test; $P < 0.05$; see Figure 1.1). As expected, HMR and BMR were significantly different among the three smaller trochilid species, (t-test, $P < 0.0001$ for each species; see Table 1.2).

For pooled data from hummingbird species excluding the giant hummingbird, BMR scaled positively with body mass ($BMR \propto M^{0.81}$; see Fig. 1.2 A, dashed line). The observed BMR (0.32 W; see Table 1.1) for the Giant Hummingbird was within the 95% confidence interval (CI = 0.17 - 0.51 W) predicted for a 20 g bird scaled upwards from small hummingbirds. Including the Giant Hummingbird datum in the BMR regression resulted in a slightly higher exponent ($BMR \propto M^{0.85}$; see Fig. 1.2 A, solid line). Similarly, the least square regression using independent contrasts showed a significant relationship between BMR and body mass ($BMR \propto M^{0.85}$; see Fig. 1.2 B) when we included the Giant Hummingbird. DEE increased more gradually with body mass when the datum for the Giant Hummingbird was included ($DEE \propto M^{1.01}$) than when it was excluded ($DEE \propto M^{1.20}$; see Fig. 1.2 C). As seen with BMR, the observed DEE for the Giant Hummingbird (1.58 W; see Table 1.1) was within the 95% confidence interval (CI = 1.22 - 3.22 W) predicted from small hummingbirds. DEE and body mass were also positively correlated in the independent contrasts analysis ($DEE \propto M^{1.01}$; Fig. 1.2 D). As with both BMR and DEE, HMR scaled more gradually with body mass when the Giant Hummingbird was included ($HMR \propto M^{0.78}$) than when this taxon was excluded ($HMR \propto M^{0.82}$; Fig. 2E). Again, the observed HMR for the Giant Hummingbird (2.92 W; see Table 1.1) was within the 95% confidence interval (CI = 2.25 - 4.57 W) predicted from small hummingbirds. Although the independent contrasts analyses showed a significant relationship between hovering metabolic rate and body mass, the slope was higher compared to the slope for the species mean values ($HMR \propto M^{0.91}$; Figure 1.2 F).

Overall for hummingbirds (including data for the Giant Hummingbird), allometric regression slopes for BMR, DEE, and HMR not incorporating the effects of phylogenetic similarity did not differ significantly ($F = 1.7$, d.f. = 2, $P = 0.20$; see Fig. 1.3). This result suggests that energetic expenditure during the different activities studied here are constant multiples of BMR across the full size range of hummingbirds.

Discussion

These measurements of metabolic rates, along with allometric analysis of energetics in the Trochilidae, enable assessment of possible outlier status in the Giant Hummingbird along with a broader test of body size limitations on hummingbird physiological capacity.

Energy expenditure in the Giant Hummingbird

As expected, mean values of HMR were higher than all other rates of energetic expenditure in the Giant Hummingbird (see Fig. 1.1). Values of BMR for this species as measured in this study (0.32 W) were comparable to those obtained by Lasiewski et al. (1967), for which the average BMR from three individuals was 0.29 W. Values of BMR did not deviate substantially from the allometry predicted by data for ten smaller species (2.8 – 7.3 g body mass; see Fig. 1.2 A, B). The maximum metabolic scope (i.e., the ratio of HMR to BMR) obtained here for the Giant Hummingbird was 10.3. The sustained metabolic scope (i.e., the ratio of DEE to BMR) was, by contrast, substantially lower (5.6). In two smaller species of hummingbirds (*Archilochus alexandri* and *Calypte anna*, weighing 3.4 g and 4.2 g, respectively), sustained metabolic scopes, as estimated from the literature and measured in free-ranging individuals by different authors, were substantially lower (4.3 and 4.1, respectively) (DEE: Powers and Nagy 1988, Powers and Conley 1994; BMR: Lasiewski 1963). In general, sustained metabolic scopes greater than seven are thought to be physiologically unsustainable for vertebrates (Hammond and Diamond 1997). Estimates for both the Giant Hummingbird and the two smaller species are below this hypothetical limit. Nevertheless, the metabolic scope for the Giant Hummingbird is higher when compared to those of the smaller species.

However, it is also possible that values of DEE measured here for the Giant Hummingbird in an enclosure may underestimate actual performance in the wild. Nagy et al. (1999) provided an allometric equation for free-ranging DEE in five species of hummingbirds (mass range from 3.7 - 8.8 g). For a hypothetical mass of 17.5 g corresponding to the Giant Hummingbird (Table 1.1), this allometry estimates a DEE of 2.1 W, which is 30% higher than the value obtained here (see Table 1.1), but is also within the 95% confidence intervals of the measured value (as estimated from ordinary least squares regression, CI = 1.22-3.22 W). Estimates for DEE obtained here may be reduced relative to free-ranging performance, as measurements here were made on solitary individuals confined within an aviary and provided nectar *ad libitum*. Stiles (1971) showed that Anna's Hummingbirds in captivity spend only half as much time flying as they do in the wild, and metabolic expenditures of Giant Hummingbirds in an aviary might also be reduced. On the other hand, captivity may increase stress in the individuals, which could counteract the decrease in activity as suggested by Stiles (1971). Nevertheless, using the extrapolated value of DEE from Nagy et al. (1999) for a hypothetical Giant Hummingbird, the sustained metabolic scope would be approximately 7.2, i.e., slightly in excess of the theoretical maximum proposed by Hammond and Diamond (1997). Doubly-labelled water studies of metabolic rates in the field would be necessary to unequivocally assess sustained metabolic scope in this over-sized species.

Metabolic rates in hovering flight

For nine hummingbird species ranging in body mass from 3 – 10 g, Voigt and Winter (1999) concluded that HMR scaled with mass$^{0.93}$, and suggested accordingly that the mass-specific metabolic rate during hovering was independent of size. Here, when data for the Giant Hummingbird and the three additional species are included, HMR scales with mass$^{0.78}$ (Fig 1.2 E; solid line). Mass-specific metabolic rate in hovering declines somewhat with increasing mass (i.e., in proportion to mass$^{-0.21}$, $P = 0.028$). Similarly, use of independent contrasts predicts a steeper, however, not significant decline in mass-specific hovering metabolic rate, in proportion to mass$^{-0.62}$ (log HMR ($mlO_2/g/h$) = -0.62 log M, $r^2 = 0.08$, $P = 0.38$). The regression of Voigt and Winter (1999) predicts a value of HMR of 3.9 W for a 17.5 g hummingbird, which is about 33% higher than that found here (see Table 1), but which is also within 95% confidence intervals of the measured value (based on ordinary least squares regression, (CI = 2.25 - 4.57 W). Values of HMR predicted from the regression of Voigt and Winter (1999) for a hummingbird with a mass similar to that of *O. estella* (~7.5 g) are similarly higher by about 48% from those actually obtained here. It is possible that their lower metabolic rates in hovering flight, in these two high-elevation species, derive from an adaptation for reducing the cost of flight at high elevations.

All measurements of HMR for the four study species were made at a common elevation of ~500 m.a.s.l. Two of the four Giant Hummingbirds studied were captured at an elevation of 3580 m.a.s.l., but mean values of HMR for these individuals were very similar to those of the other four *P. gigas* obtained at 1100 m.a.s.l. (i.e., mean values of 3.0 W vs. 3.1 W). Because hummingbird flight performance is progressively challenged at higher elevations (Altshuler and Dudley 2002; Altshuler and Dudley 2006), a study of hovering metabolism in *P. gigas* at different elevations would be of particular interest. Two other hummingbird species studied here (*O. estella* and *R. vesper*) are also high-elevation species, and values of HMR reported here may be somewhat lower than would characterise flight at their capture elevation. The allometry reported by Voigt and Winter (1999) derives from metabolic data on nine hummingbird species obtained at or near sea-level and provides a reasonable comparison to the data reported here for hovering at ~500 m.a.s.l., corresponding ~95% of sea-level air density. Maximum load-lifting performance by hummingbirds is known to degrade at higher altitudes (Altshuler et al. 2004), but general interspecific patterns of both hovering and maximal metabolic capacity are currently unknown across elevational gradients.

Energetic limits to hummingbird body size

The Giant Hummingbird exceeds in body mass the next largest hummingbird species by an approximate factor of two. It is nonetheless striking that the allometric relationships between BMR, HMR, and DEE among hummingbirds exhibit comparable scaling exponents. Given parallel allometries of HMR and BMR, metabolic scopes (i.e., the ratio of HMR to BMR) are relatively constant (~12) over a body size range of an order of magnitude. No obvious constraint to hummingbird body size emerges from these allometric considerations. Hovering *per se*, however, does not necessarily correspond to maximum metabolic rate during flight, which may occur in other behavioural contexts (e.g., flight in hypodense air or fast forward flight; see Chai and Dudley 1995; Clark and Dudley 2009). It is certainly possible that the Giant Hummingbird, particularly when hovering at high elevations (i.e., 4500 m.a.s.l.), is close to its maximum

metabolic capacity. Any increases in body size would then further degrade the power reserve necessary for behaviours supplemental to hovering. Therefore, the evolution of larger hummingbird species may be correspondingly restricted to lower elevations. The decrease in power reserves can only be assessed by direct measurements of hovering metabolic rates at high altitude for this taxon, together with imposed aerodynamic (e.g., hypodense manipulations) and physiological challenges (e.g., hypoxia) under presumed limiting conditions.

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Tables and Figures

Table 1.1: Body mass and metabolic rates (mean ± 1 s.d.) of the Giant Hummingbird under four different measurement conditions (sample size *N*): BMR, basal metabolic rate; DEE, daily energy expenditure; HMR, hovering metabolic rate.

Energetic measurement	Body mass (g)	Metabolic rate (Watts)
BMR ($N = 6$)	19.96 ± 3.20	0.32 ± 0.08
DEE ($N = 4$)	17.45 ± 2.03	1.58 ± 0.59
HMR ($N = 4$)	17.5 ± 2.12	2.92 ± 0.20

Table 1.2: Body mass and metabolic rates (mean ± 1 s.d.) for three additional hummingbird species (sample size *N*) under two measurement conditions: BMR, basal metabolic rate; HMR, hovering metabolic rate.

	Rhodopis vesper	*Sephanoides sephaniodes*	*Oreotrochilus estella*
mass (g)	4.75 ± 0.51 (*N* = 6)	5.05 ± 0.90 (*N* = 7)	7.33 ± 0.48 (*N* = 5)
BMR (W)	0.11 ± 0.02	0.10 ± 0.02	0.12 ± 0.01
mass (g)	4.20 ± 0.31 (*N* = 4)	5.54 ± 0.68 (*N* = 5)	7.92 ± 0.36 (*N* = 2)
HMR (W)	0.87 ± 0.13	1.21 ± 0.29	1.21 ± 0.20

Figure Legends

Figure 1.1: Metabolic rates of the Giant Hummingbird (mean ± 1 s.d.) under three different measurement conditions. Different letters above the bars indicate significant differences between rates (one-way ANOVA, $F_{2,24} = 130.53$, $P < 0.0001$; *a posteriori* Tukey test, $P < 0.05$).

Figure 1.2: Allometric scaling of energy expenditure in hummingbirds. Plots on the left (*a*, *c*, *e*) indicate log-log regressions of metabolic rate versus body mass using mean values by species. Dashed lines indicate a regression including all species except for the Giant Hummingbird, whereas the continuous line is the best fit for all taxa. Plots on the right (*b*, *d*, *f*) indicate regressions for the same variables using standardized independent contrasts for all taxa. In (*a*) and (*c*), values for BMR and DEE, respectively, are those measured in this study (Table 1) and data in Nagy *et al*. (1999) and Lasiewski (1963). In (*e*), values for HMR include those measured in this study (Tables 1 and 2) and those from Wolf and Hainsworth (1971), Berger and Hart (1972), Berger (1974), Epting (1980), Bartholomew and Lighton (1986), Suarez et al. (1990), and Chai and Dudley (1996).

Figure 1.3: Allometric scaling of energetic activities among *N* different hummingbird species. Symbols as follows: closed circles, BMR; open circles, DEE; triangles, HMR. Regression equations (Energy (W) = *a* mass $(g)^b$) as follows: BMR ($N = 11$), $a = 0.025$, $b = 0.85$, $R^2 = 0.94$, $P < 0.001$; DEE ($N = 6$): $a = 0.081$, $b = 1.01$, $R^2 = 0.97$, $P < 0.003$; HMR ($N = 12$): $a = 0.326$, $b = 0.78$, $R^2 = 0.82$, $P < 0.0001$).

Figure 1.1

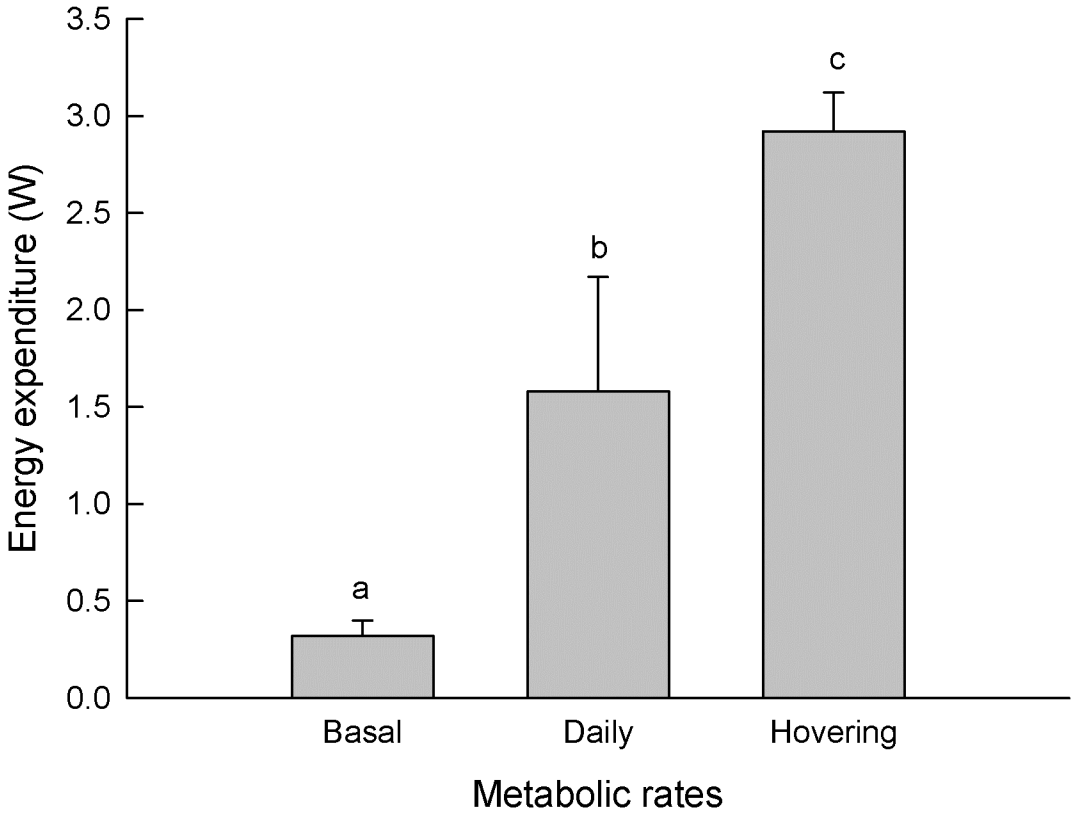

3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
Energy expenditure (W)
a
b
c
Basal
Daily
Hovering
Metabolic rates

Figure 1.2

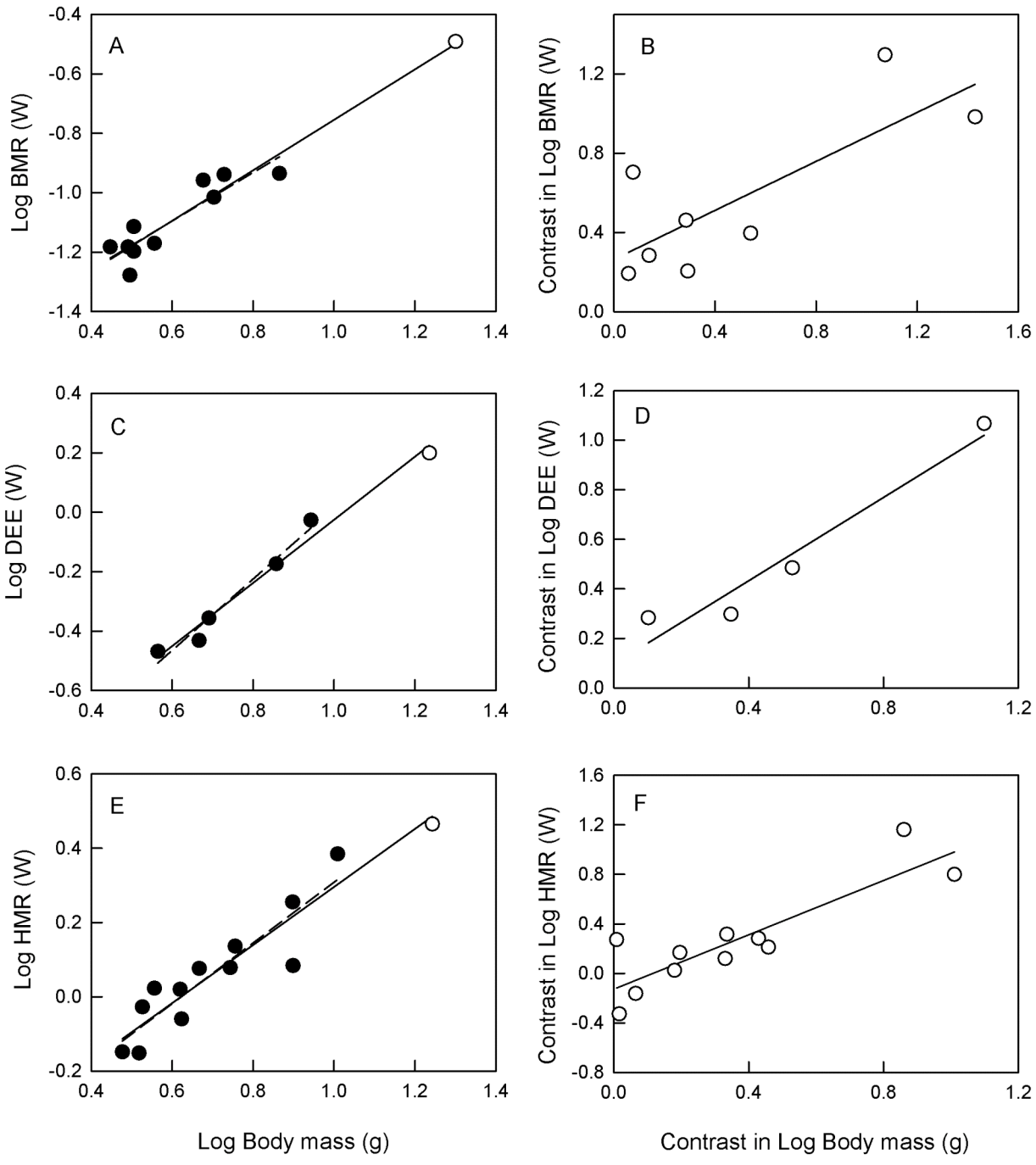
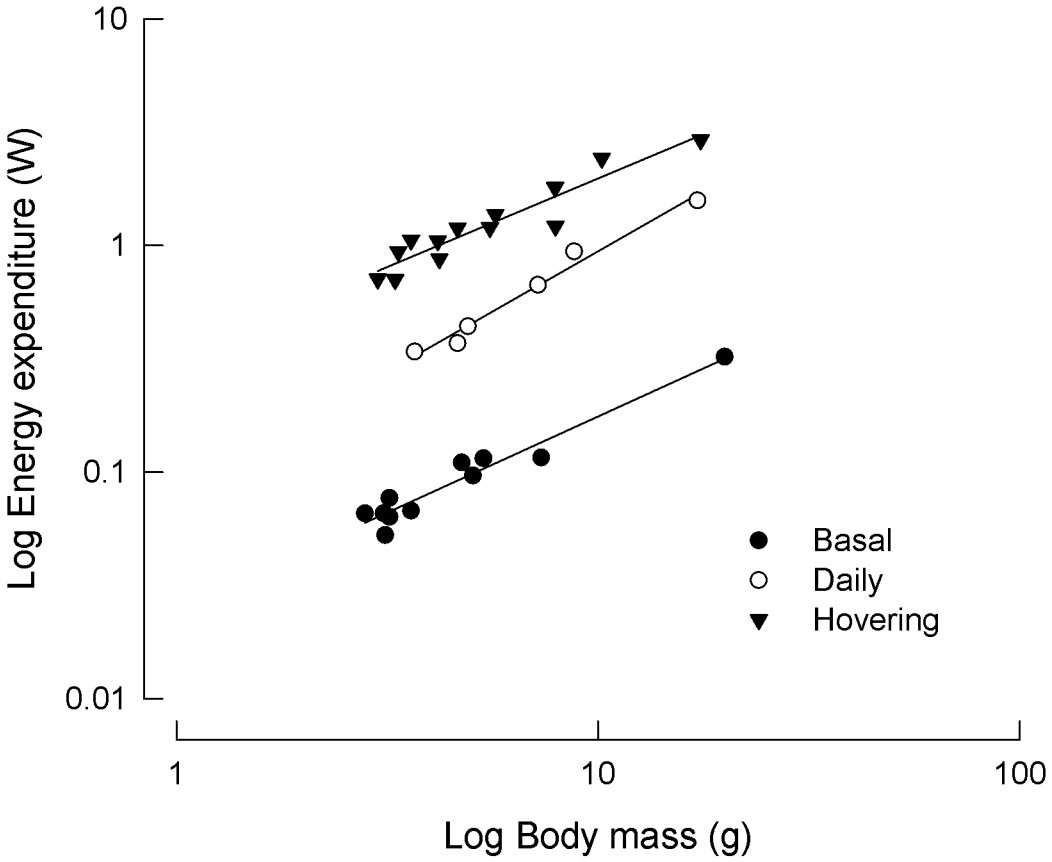

A
B
C
D
E
F
Log BMR (W)
Contrast in Log BMR (W)
Log DEE (W)
Contrast in Log DEE (W)
Log HMR (W)
Contrast in Log HMR (W)
Log Body mass (g)
Contrast in Log Body mass (g)

Figure 1.3


10
1
0.1
0.01
Log Energy expenditure (W)
1
10
100
Log Body mass (g)
Basal
Daily
Hovering

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Chapter2

Enzymatic flux capacities in hummingbird flight muscles: A "One Size Fits All" Hypothesis

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Abstract

Hummingbirds (family Trochilidae) are among the smallest endothermic vertebrates representing an extreme, among birds, in their physiological design. They are unique in their ability to sustain hovering flight, one of the most energetically demanding forms of locomotion. Given that hovering metabolic rate (HMR) in hummingbirds scales allometrically as mass$^{0.78}$, we tested the hypothesis that variation in HMR may be correlated with variation in maximal enzyme activities (V_{max} values) of key enzymes in glucose and fatty acid oxidation pathways in the flight muscles of four species of hummingbirds ranging in body mass from 4 to 20 grams. We also estimated metabolic flux rates from respirometric data obtained during hovering flight. The data are striking in the lack of correlation between V_{max} values and flux rates at most steps in energy metabolism, particularly at the hexokinase and carnitine palmitoyltransferase reactions. In the context of *hierarchical regulation analysis*, this finding suggests that *metabolic regulation* (resulting from variation in substrate, product or allosteric effector concentrations) dominates as the proximate explanation for the interspecific variation in flux. On the other hand, we found no evidence of *hierarchical regulation*, i.e., variation in V_{max} based on variation in enzyme concentration [*E*] and subject to regulation by gene expression. The evolutionary conservation of pathways of energy metabolism suggests that "one size fits all" among hummingbirds.

Introduction

Hummingbirds (Trochilidae) are characterised by their small size, extreme physiological design and extraordinary aero-acrobatics. Trochilidae represent one of the largest avian families, comprising 330 species that weigh between 2 (Cuban Bee Hummingbird, *Mellisuga helenae*) and 22 grams (Giant Hummingbird, *Patagona gigas*) (Dunning 1993; Cotton 1996; Dickinson 2003). Hummingbirds are unique among birds in their capacity for sustained hovering flight, which is one of the most energetically demanding forms of locomotion (Weis-Fogh 1972). During hovering (Lasiewski 1963; Bartholomew and Lighton 1980; Suarez 1992; Clark and Dudley 2009) and fast forward flight (Berger 1985; Clark and Dudley 2009; Clark and Dudley 2010), they display some of the highest mass-specific rates of metabolism known among vertebrate animals. Even higher metabolic rates are achieved in other contexts (e.g., flight in hypodense air; see Chai and Dudley 1995). During flight, 90% or more of whole-body VO_2 (rate of oxygen consumption) and VCO_2 (rate of CO_2 production) values are accounted for by flight muscle mitochondria (Suarez 1992; Taylor 1987). Therefore, under steady-state conditions, gas-exchange rates can be used to estimate rates of muscle ATP turnover as well as flux rates through pathways of substrate oxidation (Brand 2005; Suarez et al. 1990).

Previous studies on rufous (*Selasphorous rufus*), Anna's (*Calypte anna*) and broad-tailed hummingbirds (*Selasphorus platycercus*) revealed that recently-ingested sugar directly fuels

hovering metabolism in fed individuals; in contrast, fasted hummingbirds rely mainly on fatty acid oxidation (Suarez et al. 1990; Welch et al. 2006; Welch et al. 2008). The high rates of sugar and fatty acid oxidation during flight are made possible by high enzymatic flux capacities in the pectoral muscles (Suarez et al. 1986; Suarez et al. 1990). However, such biochemical data have been obtained only from rufous hummingbirds. Given the range of body sizes among hummingbirds, an important question is whether there might be interspecific variation in the enzymatic capacities for substrate oxidation. A recent study investigating scaling relationships between energy expenditure and body mass in hummingbirds, showed that hovering metabolic rates scale allometrically as $mass^{0.78}$ among species ranging from 3 to 20 grams (Fernández (Chapter 1); M.J. Fernández, R. Dudley and F. Bozinovic (submitted)). In addition, not all hummingbird species engage in hover-feeding behaviour; some species perch while drinking floral nectar (Carpenter 1976).

In principle, interspecific variation in metabolic rates, e.g., during hovering, as in the present context, might be based on variation in flux capacities in bioenergetic pathways, measurable *in vitro* as the maximal enzyme activities, V_{max} values of metabolic enzymes (Newsholme and Crabtree 1986; Suarez 1996; Darveau et al. 2005). Variation in V_{max} results from variation in enzyme concentration, $[E]$, given the relationship $V_{max} = [E] \times k_{cat}$, where k_{cat} is the catalytic efficiency or turnover number of each enzyme molecule (in interspecific studies of animals with similar body temperatures, k_{cat} values of enzyme orthologs can be assumed to be invariant (Hochachka and Somero 2002). Alternatively, enzyme concentrations may not vary interspecifically; instead, interspecific variation in metabolic rates may be based on metabolic regulation resulting from variation in substrate, product or allosteric effector concentrations.

In this study, we tested the hypothesis that the qualitative "design" of pathways of energy metabolism (Suarez et al. 1990) is highly conserved among hummingbirds. In addition, we hypothesize size-related, quantitative variation in V_{max} values that may partly account for the allometry of hovering metabolic rates. We measured, *in vitro*, the V_{max} values of key enzymes in pathways of glucose and fatty acid oxidation in the flight muscles of four species of hummingbirds found along the Andes (*Rhodopis vesper*, *Sephanoides sephaniodes*, *Oreotrochilus estella*, and the Giant Hummingbird *Patagona gigas*) ranging in body mass from 4 to 20 grams. These species cover 88% of the full range of body masses among the Trochilidae. Like all other hummingbirds these species are obligate nectarivores and engage in hovering-feeding behaviour, except for the Andean Hillstar, *O. estella*. This species inhabits exclusively high elevations (above ~3000 m.a.s.l.) and often perches to obtain the nectar; it even breaks off flowers to drink nectar while on the ground (Carpenter 1976; M.J. Fernández (personal observation)). We also estimated metabolic flux rates from respirometric data obtained during hovering flight (Fernández (Chapter 1); M.J. Fernández, R. Dudley and F. Bozinovic (submitted)). The data are interpreted in the context of *hierarchical regulation analysis* (ter Kuile and Westerhoff 2001; Suarez et al. 2005), which makes the distinction between variation in flux based on variation in $[E]$, referred to as "*hierarchical regulation*" (resulting from variation in some aspect of gene expression), or based on *metabolic regulation* resulting from mass-action or allosteric effects.

Materials and Methods

Animals, Tissue Sampling and Storage

Hummingbirds were captured in Chile using mist nets between March - July 2002. *Patagona gigas* (n = 4), *Oreotrochilus estella* (n = 4), and *Rhodopis vesper* (n = 4) were captured in northern Chile, Chusmiza (19° 40´ S, 69° 10´ W, ~3583 m), and *Sephanoides sephaniodes* (n = 3) were captured in central Chile (33° 17' S, 71° 11' W, ~600 m.a.s.l.). Hummingbirds were transported to the Pontificia Universidad Católica de Chile in Santiago (33º 27' S, 70º 40' W, ~520 m.a.s.l.) where they were euthanized by thoracic compression. Flight muscles were dissected out, immediately frozen in liquid N_2 and stored in cryovials at -80 °C until transported in dry ice to the University of California, Santa Barbara, USA. Flight muscles were stored at -80 °C until 2008 when maximal enzyme activities were measured.

Tissue preparation for enzyme assays

Tissue preparation was implemented as described by Suarez et al. (2009). Muscles (~40 mg) were minced with fine scissors and homogenized, using a Pro 200 homogenizer (Pro Science, Oxford, CT, USA), in 9 volumes of homogenizer buffer. The homogenizer buffers used in this study where: 25 mM HEPES (pH 7.0), 50 mM imidazole (pH 7.1), 50 mM Tris-Cl (pH 7.2) or 50 mM sodium phosphate (pH 7.4) with 2 mM EDTA, 0.5% (v/v) Triton X-100 and 5 mM β-mercaptoethanol (added before homogenization), except in the case of assays requiring 5,5' – dithiobis (2- nitrobenzoic acid), (i.e., in citrate synthase (CS) and carnitine palmitoyltransferase (CPT) assays). The indicated pH values were measured at room temperature. Homogenates were sonicated using a Microson Ultrasonic Cell Disruptor model # MS-50 (Heat Systems Ultrasonics Inc., Farmingdale, NY, USA) and then centrifuged for 4 min at 10,000 rpm at 4 °C using an IEC Micromax refrigerated microcentrifuge (Needham Heights, MA, USA). Supernatant fractions in microcentrifuge tubes were kept in ice until assays were completed.

Maximum Enzyme Activities

Assay buffers used in this study were the same as reported by Suarez et al. (2009). We measured maximal enzyme activities (V_{max}) of seven enzymes involved in the glucose and fatty acid oxidation pathways. Four enzymes participate in carbohydrate oxidation: i) Glycogen phosphorylase (GP), catalyzes the degradation of glycogen, ii) hexokinase (HK) is responsible for catalyzing glucose phosphorylation and entry into the glycolytic pathway, iii) phosphofructokinase (PFK), is an allosteric enzyme that may play a role in the control of glycolysis, iv) lactate dehydrogenase (LDH), catalyzes a near-equilibrium reaction that, under certain conditions, leads to net lactate production. Two mitochondrial enzymes involved in the fatty acid oxidation pathway were measured: i) 3-hydroxyacylCoA dehydrogenase (HOAD) is an enzyme in the β-oxidation pathway, and ii) carnitine palmitoyltransferase (CPT) is an enzyme that mediates the transport of long-chain fatty acid across the mitochondrial membrane by catalysing transesterification reactions with coenzyme A and carnitine. We measured citrate synthase (CS), which catalyzes the first reaction in the Krebs cycle and serves as a mitochondrial marker (Moyes 2003). The assays were done in duplicate, as described by Suarez et al. (1986).

We used a Shimadzu UV-160U recording spectrophotometer at 39 °C; temperature was maintained using a Grant circulating water bath (model LTD 6, Cambridge, UK). Each enzyme measurement had a control (background) obtained without one substrate. Control rates were measured and subtracted from rates obtained with all substrates present. We varied substrate concentrations to obtain V_{max} values, ensuring that substrates were saturating and not inhibitory. Coupling enzymes, where needed, were added in excess; this was verified by varying the activities added to assay mixtures.

Data analysis

Difference in enzyme activities between species were analysed using a Kruskal-Wallis test followed by a multiple comparison post-hoc test. Systematic changes in enzyme activity and flux rates *in vivo* with body mass (*M*) were tested using least squares linear regression of mean values against *M*. Differences in activity between *P. gigas* tissues collected in Chile and tissues provided by the Museum of Vertebrate Zoology at UC Berkeley (further details provided in Results section) were assessed using a Wilcoxon test. Correlation between mean flux rate *in vivo* (Fernández (Chapter 1); M.J. Fernández, R. Dudley and F. Bozinovic (submitted)) and mean flux capacity *in vitro* measured in this study was tested using a Spearman correlation test. All statistical analyses were performed using the R software (R Development Core Team 2010).

Results

To test for the effect of storage time on enzyme activity, we compared flight muscle samples (kindly provided by the Museum of Vertebrate Zoology at UC Berkeley) from three *P. gigas* collected in Peru in June - August 2006 with Chilean *P. gigas* samples collected in 2002. Mean V_{max} values were not significantly different when comparing samples collected at different times, during 2002 and 2006 (Wilcoxon test, $P > 0.05$). Enzyme activities remained stable for at least six years when stored at –80 °C, except for PFK, which was not detectable in any of the tissues.

Mean values for maximum enzyme activities in four trochilid species, ranging in body mass from 4.3 to 19.6 grams, are given in Table 2.1. The mean V_{max} values for HK, LDH, CPT, and GP were not found to differ significantly between species, despite the differences in their body mass (Kruskal-Wallis test, $P > 0.05$). However, we found a significant difference in mean values of CS (Kruskal-Wallis test: 9.58, $P < 0.05$) and HOAD (Kruskal-Wallis test: 9.33, $P < 0.05$). Multiple comparison tests showed that CS activity was higher (203.3 μmol min^{-1} g wet wt^{-1}) in the smallest hummingbird, *R. vesper* (4.7 g) and lower (148.1 μmol min^{-1} g wet wt^{-1}) in the medium sized species, *S. sephaniodes* (5.5 g). However, this difference was not dependent upon body mass ($CS \propto M^{-0.07}$, $r^2 = 0.02$, $P > 0.05$). The variation in HOAD between species was only partially accounted for by body mass, which explains 23% of the variation in enzyme activity ($HOAD \propto M^{-0.16}$, $r^2 = 0.23$, $P > 0.05$). The difference was found between the giant hummingbird (16.3 g), having lower enzyme activity (231.9 μmol min^{-1} g wet wt^{-1}), and *S. sephaniodes*, with higher enzyme activity (343.3 μmol min^{-1} g wet wt^{-1}). In general, therefore, V_{max} values were largely independent of body mass.

ATP turnover and flux rates during hovering were estimated using respirometric data (Fernández (Chapter 1); M.J. Fernández, R. Dudley and F. Bozinovic (submitted)) (Table 2.2).

Muscle mass scales isometrically with body mass in hummingbirds; on average, flight muscle mass consists of 26% of total body mass (Altshuler and Dudley 2002, n=23 species; M.J. Fernández (unpublished data, n=4 species)) where 90% or more of whole-body rates of oxygen consumption occurs (Suarez 1992; Taylor 1987). Assuming that only glucose is oxidised and a P/O ratio (ATP molecules synthesized/O atom consumed) of 2.41 (Brand 2005), mean ATP turnover rate for *R. vesper, S. sephaniodes*, *O. estella* and *P. gigas* would be 461.3 ± 22.9, 480.9 ± 30.4, 333.2 ± 22.3 and 356.1 ± 15.7 μmol g^{-1} min^{-1} (mean ± s.e.) respectively. To sustain the above rates of ATP turnover, hummingbirds requires the oxidation of glucose at a rate between 10.4 to 19.0 μmol g^{-1} min^{-1}, and the oxidation of fatty acids at a rate between 2.0 to 3.7 μmol g^{-1} min^{-1}. Mean values for glucose and palmitate oxidation rates during hovering flight are given in Table 3. For both substrates the oxidation rates differ significantly between species (Kruskal-Wallis test, $P < 0.05$). Specifically, *S. sephaniodes* require oxidation rates 44% higher on both substrates (glucose and palmitate) compared to *O. estella* (Table 2.3). Through a least square linear regression we found that body mass accounted for 33% of the variability in flux rates through the glucose and long-chain fatty acid oxidising pathways (flux rates $\propto M^{-0.19}$, $r^2 = 0.33$, $P < 0.05$).

Within species, the mean body mass of the individuals used for respirometry (Fernández (Chapter 1); M.J. Fernández, R. Dudley and F. Bozinovic (submitted)) was not significantly different from those used for the enzyme V_{max} measurements (Wilcoxon test, $P > 0.05$). Given this, it is reasonable to compare steady-state flux rates at each enzymatic step (v) with enzyme V_{max} values. At the HK and CPT steps v and V_{max} were not significantly correlated (Spearman rank correlation, $P > 0.05$). If it is assumed that only glucose is oxidised during hovering, the fractional velocity, v/V_{max} at the HK step varies from 0.67 to 0.95. On the other hand, if only palmitate is oxidised, the v/V_{max} at the CPT step ranges from 0.44 to 0.84 (Table 2.3).

Discussion

Design of Pathways of Energy Metabolism

The high V_{max} values for hexokinase, carnitine palmitoyltransferase, and citrate synthase indicate high capacities for the catabolism of glucose and long-chain fatty acids, as well as high mitochondrial capacities for oxidative metabolism, consistent with the scheme proposed by Suarez et al. (1990). These high enzymatic flux capacities make possible the high rates of sugar and fatty acid oxidation (Suarez et al. 1986; Suarez et al. 1990; Welch et al. 2006), allowing hummingbird to switch between these fuels, depending on prandial state and flight behaviour. Qualitatively, the four Chilean species are identical to rufous hummingbirds (Suarez et al. 1986; Suarez et al. 1990) in terms of the overall design of pathways of energy metabolism. Also in common with rufous hummingbirds, in the Chilean species, hexokinase and CPT operate at high fractional velocities (v/V_{max}) during hovering flight when either glucose or fatty acid is oxidised (Table 2.3). Thus, the high rates of glucose and fatty acid oxidation during flight are achieved through high capacities for flux as well as the operation of key enzymes at high fractional velocities (Suarez et al. 1990). Hummingbird flight muscle LDH activities are lower than in other avian species, especially those that possess flight muscles consisting of fast-twitch, glycolytic fibers (Crabtree and Newsholme 1972), indicating low capacities for anaerobic

glycolysis. This is consistent with the design of their fast-twitch, oxidative fibers for highly aerobic exercise (Grinyer and George 1969; Suarez et al. 1991, Welch and Altshuler 2009).

Maximum Capacities for Flux

A recent study (Fernández (Chapter 1); M.J. Fernández, R. Dudley and F. Bozinovic (submitted)) showed that HMR scales positively with body mass (HMR $\propto M^{0.78}$); consequently, mass-specific HMR scales negatively with mass (HMR/$M \propto M^{-0.21}$). Given the influence of body mass on rates of energy expenditure (McNab 2002) and the allometric scaling of hovering metabolic rates among hummingbirds, it is reasonable to hypothesize the occurrence of mass-dependent variation in enzymatic flux capacities in pathways of energy metabolism. Allometry in the V_{max} values of oxidative enzymes has been observed previously in the locomotory muscles of pelagic fishes (Somero and Childress 1990) and mammals (Emmett and Hochachka 1981). It was therefore somewhat surprising to find no interspecific variation among hummingbirds in V_{max} values for most of the enzymes involved in glucose and fat oxidation pathways, except for CS and HOAD. In the case of HOAD, 23% of the variability in V_{max} could be explained by body mass, where *S. sephaniodes*, a medium-sized hummingbird (5.5 g) had the highest V_{max} activities and *P. gigas*, the largest extant species (16.3 g), had the lowest values. On the other hand, body mass did not explain the variation in the V_{max} values of CS. Given the use of CS as an index of mitochondrial content (Suarez et al. 1991; Moyes 2003), our results suggest the absence of a consistent pattern of mass-dependent variation in mitochondrial content in hummingbirds.
The enzymatic flux capacities at the CS and HOAD reactions are far greater than flux rates through the citric acid cycle and fatty acid oxidation, respectively. We are aware of no evidence in the biochemical literature that these enzymes exert significant control over these pathways. In contrast, there is some evidence from metabolic control analysis that CPT may have significant control over fatty acid oxidation (Spurway et al. 1997; Eaton et al. 2001). Hexokinase plays a significant regulatory role over glucose oxidation in exercising muscles (Fueger et al. 2004). Thus, the observed interspecific variation in V_{max} values for CS and HOAD may have little or no functional significance. Our data do not support the hypothesis that quantitative "biochemical adaptation" in flux capacities has occurred within trochilids. Instead, suggest that "one size fits all", i.e., that enzymatic flux capacities are not quantitatively adjusted in relation to interspecific variation in body mass. This implies that interspecific variation in flux through pathways of glucose and fatty acid oxidation is achieved through modulation of enzyme activities, rather than adjustments in [*E*] resulting from interspecific variation in gene expression.

Metabolic regulation dominates over hierarchical regulation

We showed that V_{max} values at the hexokinase and carnitine palmitoyltransferase steps are independent of body mass, while flux rates at both steps, estimated from respirometric data, showed negative allometric scaling with body mass (mass explains 33% of the variability in oxidation rates). The lack of a positive correlation between flux rates and enzyme V_{max} values suggests that variation in flux is not due to "hierarchical regulation" but instead is due to "metabolic regulation" at the HK and CPT steps, in the scheme proposed by ter Kuile and Westerhoff (2001) and applied by Suarez et al. (2005) to the allometric variation in flight metabolic rates in Panamanian orchid bees.

On the other hand, muscle power output would be expected to drive the interspecific variation in metabolic rates. Wingbeat frequency and stroke amplitude are the most important mechanisms that modulate muscle power (Ellington 1984). Altshuler and Dudley (2003) showed that wingbeat frequency decreases as mass$^{-0.21}$, while stroke amplitude is independent of body mass (Altshuler et al. 2010). If we make the assumption that, across hummingbird species, muscle stress and strain scale isometrically, then we would expect muscle volume-specific (or mass-specific) power output (Pennycuick and Rezende 1984) to scale negatively with mass. Since muscle power output determines the steady-state rates of ATP turnover during exercise and oxidative pathways support aerobic ATP turnover rates, then it follows that mass-specific power output would drive the scaling of metabolic rate. These relationships likely underlie the finding that body mass explains 33% of the variation in rates of ATP turnover, as well as glucose and palmitate oxidation rates. The rates at which muscles perform mechanical work determine their ATP hydrolysis rates, while these drive rates of ATP synthesis by oxidative pathways and mitochondrial O_2 consumption drives O_2 flux through the cardio-respiratory system from the external environment.

Hierarchical regulation involves the variation of flux via alterations in V_{max}, based on variation in [*E*]. It is assumed that this results from variation in some aspect of gene expression (ter Kuile and Westerhoff 2001). The data presented here are inconsistent with hierarchical regulation of flux at the metabolic steps examined. On the other hand, metabolic regulation involves variation in flux resulting from modulation of enzyme activities by the concentrations of substrates, products or allosteric effectors. In hierarchical regulation analysis (ter Kuile and Westerhoff 2001), when hierarchical regulation is ruled out, metabolic regulation remains as the working hypothesis. Thus, we propose that over an evolutionary time-scale, hummingbirds retained a highly conserved set of pathways for muscle energy metabolism. Both qualitatively as well as quantitatively, "one size fits all", and interspecific variation in flux in pathways of energy metabolism is primarily driven by variation in muscle power output. However, "one size fits all" should be regarded as a working hypothesis and tested further with a larger number of species using the method of phylogenetically independent contrasts (Felsenstein 1985).

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Tables

Table 2.1: Body mass in grams and maximum enzyme activities (mean ± s.e.) in flight muscles of four hummingbird species (sample size in parentheses). V_{max} values are expressed in μmol min^{-1} g wet wt^{-1}.

Enzyme	*R. vesper*	*S. sephaniodes*	*O. estella*	*P. gigas*
Body mass	4.7 ± 0.2 (4)	5.5 ± 0.5 (3)	7.6 ± 0.2 (4)	16.3 ± 1.3 (4)
Hexokinase	22.3 ± 1.0 (4)	17.5 ± 1.3 (3)	17.3 ± 1.3 (4)	17.3 ± 2.8 (4)
Lactate dehydrogenase	275.7 ± 8.2 (4)	378.2 ± 31.0 (3)	275.8 ± 22.5 (4)	268.7 ± 9.2 (4)
Carnitine palmitoyltransferase	4.7 ± 0.3 (4)	3.9 ± 0.8 (3)	5.1 ± 0.7 (4)	4.0 ± 0.1 (4)
Citrate synthase	203.3 ± 7.4 (4)	148.1 ± 13.6 (3)	153.7 ± 10.1 (4)	172.4 ± 9.7 (4)
Hydroxyacyl-CoA dehydrogenase	258.5 ± 23 (4)	342.3 ± 18.7 (3)	291.8 ± 11.3 (4)	231.9 ± 4.5 (4)
Glycogen phosphorylase	32 ± 2.9 (4)	54.4 ± 4.8 (3)	34.4 ± 3 (4)	31.9 ± 0.7 (4)

Table 2.2: Body mass (g) and mass-specific metabolic rate during hovering flight, HMR (mean ± s.e.) for four hummingbird species (sample size in parentheses). Data adapted from Fernández et al. (submitted). HMR values are expressed in $mlO_2\ g^{-1}\ h^{-1}$.

	R. vesper	*S. sephaniodes*	*O. estella*	*P. gigas*
Body mass	4.2 ± 0.1 (5)	4.7 ± 0.2 (5)	7.5 ± 0.3 (3)	18.7 ± 1.5 (3)
HMR	37.1 ± 1.8	38.7 ± 2.4	26.8 ± 1.8	28.7 ± 1.3

Table 2.3: Required substrate oxidation rates, maximum possible rates of flux and fractional velocities (v/V_{max}) through hexokinase and carnitine palmitoyltransferase in flight muscles of four hummingbird species (sample size in parentheses) during hovering flight (mean ± s.e.).

	Oxidation rate (μmol g^{-1} min^{-1})		
Glucose oxidation	Required	Maximum possible	v/V_{max}
			Hexokinase
R. vesper	16.0 ± 0.8 (5)	22.3 ± 1.0 (4)	0.72
S. sephaniodes	16.6 ± 1.0 (5)	17.5 ± 1.3 (3)	0.95
O. estella	11.5 ± 0.8 (3)	17.3 ± 1.3 (4)	0.67
P. gigas	12.3 ± 0.5 (4)	17.3 ± 2.8 (4)	0.71

	Oxidation rate (μmol g^{-1} min^{-1})		
Palmitate oxidation	Required	Maximum possible	v/V_{max}
			CPT
R. vesper	3.1 ± 0.1 (5)	4.7 ± 0.3 (4)	0.66
S. sephaniodes	3.2 ± 0.2 (5)	3.9 ± 0.8 (3)	0.84
O. estella	2.2 ± 0.1 (3)	5.1 ± 0.7 (4)	0.44
P. gigas	2.4 ± 0.1 (4)	4.0 ± 0.1 (4)	0.60

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Chapter 3

Elevational variation in flight kinematics and energetics of the Giant Andean Hummingbird (*Patagona gigas*)

Abstract

Patagona gigas (Giant Hummingbird) weighs on average 20 grams, almost twice as much as the second-largest hummingbird. Despite the challenges of supporting its large body weight, *P. gigas* inhabits a broad altitudinal range (sea level to 4500 m) along the Andes in South America. Relative to sea level, high elevation environments present challenges to flying animals. At high elevations, the lower air density relative to sea level demands higher mechanical power output. Furthermore, the lower partial pressure of oxygen may constrain metabolic power production thereby decreasing the oxidative capacities in organisms. We explored the mechanisms used by the Giant Hummingbird to cope with the enhanced energetic and aerodynamic demands of living at high elevations. We measured flight kinematics (wingbeat frequency and stroke amplitude) and energetics (oxygen consumption) during hovering flight at two elevations spanning a 3750 m elevational gradient along the Andes Mountains of Peru. Contrary to our predictions, *P. gigas* increased wing stroke amplitudes and wingbeat frequencies equally, and thus increased mechanical power output at high elevations relative to sea level. Moreover, oxygen consumption during hovering increased significantly (~33%) at the high elevation site. However, based on the observed increase in mechanical power, the 33% increase in metabolic power was larger than expected.

Introduction

Hummingbirds (Trochilidae) represent one of the largest avian families with ~330 described species (Dickinson, 2003; McGuire et al., 2007), and body masses ranging between 2 (Cuban Bee hummingbird, *Mellisuga helenae*) and 20 grams (Giant Hummingbird, *Patagona gigas)* (Dunning, 1993; Cotton, 1996). This large family is extremely diverse, not only because of their body mass distribution but also because of their ecology, behaviour, and flight related morphology (Greenewalt, 1960; Cotton, 1996; Schuchmann, 1999). Hummingbirds are only found in the New World, inhabiting broad latitudinal and altitudinal ranges (from sea level to 5000 m; Carpenter, 1976; Schuchman, 1999).

Bleiweiss (1998) suggested that hummingbirds originated in the lowlands and diversified into colder montane habitats. This hypothesis has been recently supported by McGuire et al. (2007) using a more complete phylogeny (~150 species). Thus, hummingbird adaptations to hypobaric hypoxia may have been essential for their successful colonisation of high elevation habitats. Nonetheless, diversification into higher elevations imposes a range of costs. Relative to lowland conditions, high elevation habitats represent three interrelated costs on animal flight energetics. At high elevations, reduced partial pressure of oxygen as well as decreased air temperatures pose challenges to gas exchange in flying organisms (Clemens, 1988). These

challenges may constrain metabolic power production, for example by reducing oxidative capacities. Furthermore, lower air density reduces lift production and therefore mechanical power output must increase to compensate (Ellington, 1984c).

In hovering animals, the main mechanisms for varying power demands are the modulation of the stroke amplitude (Φ) (i.e., the angular extent of wing motion within the stroke plane) and the wingbeat frequency (n) (i.e., the number of complete wingbeats per second) (Ellington, 1984c). Because hummingbirds occur across a wide range of elevations, they represent an ideal model to study the limiting factors in hovering flight performance. Modulation of wingbeat kinematics as a function of elevation has been studied previously in hummingbirds, both in the field and in laboratory conditions. Laboratory experiments have measured flight performance using gas mixtures to modify air density and oxygen partial pressures. In general, these studies have shown that hummingbirds modulate their wing stroke amplitudes as a major compensatory mechanism for increasing power production during hovering in hypodense or hypobaric environments, keeping wingbeat frequency mainly unchanged (Berger, 1974; Chai and Dudley, 1995, 1996; Chai et al., 1996). These patterns have also been observed in hovering orchid bees (Dudley, 1995). In contrast, wingbeat frequency changed slightly under hypodense normoxic air (Chai and Dudley, 1995; Altshuler and Dudley, 2003). Increasing wingbeat frequency may require a greater increase in metabolic demands than does increasing stroke amplitudes and, therefore, increasing wing stroke amplitude may be the preferred mechanism to increase lift. However, it has been documented that hummingbirds have the ability to modulate wingbeat frequency during maximum load-lifting, but only for short periods of time during anaerobic burst performance (Altshuler and Dudley, 2003). Similar to laboratory experiments, field studies measuring flight performance in hummingbird species at different elevations have shown higher wing stroke amplitudes at higher elevations while wingbeat frequency remained unchanged (Altshuler and Dudley, 2003; Altshuler et al., 2004a). However, the intra-specific variation in wingbeat kinematics and the metabolic cost as a function of elevation are currently unstudied in hummingbirds in field conditions.

Maintaining a large body mass also increases the power required to hover. Power requirements for hovering flight increase with mass $(M)^{1.17}$ (Norberg, 1995), whereas maximum aerobic capacity tends to scale negatively with M (Bishop, 1997). Thus, hovering flight is relatively more costly and metabolically challenging for heavier animals. Among hummingbirds, *Patagona gigas* (the Giant Hummingbird) is a true giant with respect to its body mass. It weighs on average 20 grams, reaching body masses of up to 25 grams. In fact, it has almost twice the mass of the next largest hummingbird (Dunning 1993; Cotton 1996; Schuchmann 1999; Dickinson 2003). Yet despite its large size, *Patagona gigas* is an obligate nectarivore and routinely engages in hovering flight, which is one of the most energetically demanding forms of locomotion requiring high levels of metabolic power input (Suarez 1992) and mechanical power output (Wells 1993). Given the metabolic, aerodynamic, and thermal challenges imposed by high elevations on the power requirements for hovering flight, it is not unreasonable to suggest that the Giant Hummingbird may face further constraints due to its large body size. Despite these challenges, *Patagona gigas* inhabits a broad altitudinal (from sea level to 4500 m; where O_2 levels are about 45% of sea level values) and latitudinal range (from southern Colombia to Argentina; see Ortiz-Crespo 1974; Schuchmann 1999). To date, two *Patagona gigas* subspecies have been recognised. *Patagona gigas peruviana* is found at mid- and high elevations, from 2000 to 4500 m, and *Patagona gigas gigas* occurs at low and mid-

elevations, from sea level to 2000 m (Schuchmann 1999). Thus, the occurrence of *Patagona gigas* at a wide range of altitudes provides an excellent "natural experiment", and the opportunity to assess behavioural, biomechanical and energetic responses of this taxon to natural hypobaric and hypoxic variations.

Here, we studied the mechanisms that allow the Giant Hummingbird to cope with the increased energetic and aerodynamic demands of living at high elevations. We measured flight kinematics (wingbeat frequency and stroke amplitude) and energetics (oxygen consumption) during hovering flight at two different elevations, separated by 3700 m, along the Andean Range in Peru. Despite its large body size, we hypothesized that Giant Hummingbirds would increase wing stroke amplitude as the main compensatory mechanism for increasing power production during hovering at high elevations. We also expect minimal changes in wingbeat frequency, as found previously in smaller hummingbird species (Berger, 1974; Chai and Dudley, 1995, 1996; Chai et al., 1996; Altshuler and Dudley, 2003). Assuming that there is a strong correlation between the supply of oxygen and the demand of the working tissues during hovering flight (Bishop and Butler, 1995; Fernández (Chapter 2); Fernández et al., *submitted*), we further hypothesized an increase in metabolic power similar to the increase in mechanical power at high elevations.

Materials and Methods

Bird capture and maintenance

Fifteen individuals (nine males and six females), of *Patagona gigas peruviana* were captured with mist nets in Carhuayuma, Departamento Lima, Peru (11º 37'S, 76º 34'W, ~3800 m) during July 2007. Measurements of kinematics and metabolic rates during hovering flight were carried out at the site of capture. Upon completion (within approximately one week), birds were transported to Lima (12º 08'S, 77º 04'W, ~50) where the same parameters were measured. Hummingbirds were maintained, between three to 10 days, in large individual cages (90 cm x 90 cm x 90 cm) with an artificial nectar solution (13% (weight/volume); Nektar-plus, Pforzheim, Germany) supplemented with (5% weight/volume) sucrose available *ad libitum*. Although the Giant Hummingbird is sexually monomorphic, we compared all the parameters measured between sexes.

Morphology, wingbeat kinematics and power requirements

Morphological parameters, for use in aerodynamic calculations (Ellington, 1984a), were determined for each individual, from photographs of the spread wings against graph paper. Images were analyzed using Image J (National Institute of Health, Bethesda, MD) to determine wing length R (mm), total wing area S (mm^2) (i.e., the area of both wings), wing loading p_w (N/m^2) (i.e. weight of the animal divided by the total wing area which correspond to the pressure exerted on the air by the wings) and aspect ratio AR (i.e., $4R^2/S$). Because it was not possible to obtain wing and muscle mass directly in the Giant Hummingbird, we estimated these measures using a least-squares linear regression obtained from data measured in 25 species of smaller hummingbirds by Altshuler (2001). The regression equations for wing mass (Mw, in g) as a

function of wing area (S, in m^2) and muscle mass (Mm, in g) as a function of body mass (M, in g) were: $Mw = 0.0001593(S)$, and $Mm = -0.414 + 0.343(M)$, respectively.

Hover-feeding hummingbirds were filmed using a mirror positioned at a 45° angle at the bottom of the cage using a high-speed digital video camera (*Motion*meter, Redlake), operating at 500 frames s^{-1} and connected to a digital video camera (Sony DCR-HC32) to save recorded videos. Video sequences were analyzed frame-by-frame to obtain values of wingbeat frequency (n) and stroke amplitude (Φ) (see Chai and Dudley, 1995, 1996). Six individuals, two females and four males, were measured in total. A minimum of six complete stroke cycles were analyzed per flight, in five flight events per individual, to calculate an average value for wingbeat frequency and stroke amplitude. Before each trial, body mass was measured using an electronic balance (Sartorius, ± 0.01 g).

Wingbeat kinematics and morphological data were coupled to calculate mechanical power output using a detailed aerodynamic model of animal hovering (Ellington, 1984) modified to incorporate current understanding of unsteady drag coefficients (Altshuler et al., 2004b). Ellington's (1984b) model for calculating aerodynamic power during hovering flight also takes into account air density and other kinematic parameters (e.g., stroke plane angle, angular velocity, and acceleration of the wing assuming simple harmonic motion). Stroke plane angle was assumed to equal zero, and simple harmonic motion was assumed for wing movements within the stroke plane (see Chai and Dudley, 1995; Altshuler and Dudley, 2003). The total aerodynamic power required by a hovering hummingbird was calculated as the sum of profile (i.e., the cost to overcome drag forces on the wings) and induced power (i.e., the power required to support body weight) assuming perfect elastic storage of wing inertial energy (Ellington, 1984c; Wells, 1993). Profile power was calculated following Ellington (1984c) using a wing drag coefficient of 0.139 (see Altshuler el al., 2004b).

Rates of oxygen consumption during hover-feeding (HMR) were obtained with an open respirometry system (see Bartholomew and Lighton, 1986) for four individuals. Hummingbirds placed their heads inside a mask attached to a feeder to access the sugar solution. Expired respiratory gases were sampled from the mask at a rate of 1000 ml/min. Water vapour and CO_2 were removed from the airstream using Drierite (Hammond, Xenia, OH) and Baralyme (Keomed, St. Paul, MN), respectively. The oxygen concentration of the incoming air was recorded with a portable oxygen analyzer (FOXBOX®, Sable Systems International Inc., Las Vegas, NV), where the oxygen depletion corresponded to the amount of oxygen consumed by the bird over the duration of the feeding bout (Bartholomew and Lighton, 1986). A photoresistor, attached to the bottom of the plastic mask, recorded the length of the feeding event. When the hummingbird's head was inside the mask (during a feeding event) the photoresistor was occluded showing a change in the voltage signal; the average duration of feeding events was 10 s, ranging from 3 – 17 seconds. Hovering metabolic rate was estimated from the volume of oxygen consumed divided by the duration of the feeding event. At both elevations a minimum of five hovering feeding bouts were recorded per individual to obtain an average value of the rate of oxygen consumption. Before each trial, body mass was measured using an electronic balance (Sartorius, ± 0.01 g). All measurements were conducted at an ambient temperature between 17 and 23 °C.

Statistics

The effects of altitude on oxygen consumption during hovering, kinematics and mechanical power were evaluated using *t*-tests for paired comparisons. Data points used in the analysis were mean values for each individual measurement at both elevations. Differences between sexes were assessed using a Wilcoxon rank sum test. Statistical analyses were performed using the R software package (R Development Core Team 2010).

Results

Mean values for body mass and morphological variables for *Patagona gigas peruviana*, separated by sex, are given in Table 3.1. In general, wing length, wing area, aspect ratio and wing loading were not significantly different between the sexes (two females and two males) for the individuals measured in this study (Wilcoxon test, $P > 0.05$). However, body mass in females (N=6) was significantly lower compared to males (N=9), 19.6 ± 0.5 and 22.6 ± 0.5 grams, respectively (Wilcoxon test, $P < 0.05$). At the same time we found no statistically significant differences (Wilcoxon test, $P > 0.05$) in wingbeat frequency and stroke amplitude (four males and two females), mechanical power output (two males and two females), and mass-specific metabolic rates (three males and two females) during hovering flight between males and females at both elevations.

Because there were no significant differences in wingbeat kinematics, morphological variables, and metabolic and mechanical power between males and females, we used a mean value for each variable obtained from both genders at both elevations. In general, wingbeat frequency, stroke amplitude, mechanical power and metabolic power differed significantly with elevation (paired *t*-test, $P < 0.05$). At high elevations *P. g. peruviana* increased its wingbeat frequency by 8% relative to lowlands (see Table 3.2 and Fig. 3.1a). At the same time stroke amplitude increased by 6% at high elevations relative to sea level (see Table 3.2 and Fig. 3.1b). In accordance with wingbeat kinematics, mechanical power output, assuming perfect elastic storage, increased by 12% at high elevation relative to sea level (see Table 3.2 and Fig. 3.2).

Mean values for hovering mass-specific metabolic rates in five individuals at the two study sites are given in Table 3.2. Relative to sea-level, the Giant hummingbird showed a 33% increase in the oxygen consumption during hovering at high elevations (see Table 3.2 and Fig. 3.3).

Discussion

Flight at high elevation is doubly challenging because of reduced air densities and oxygen availability compared to lowland habitats. Hovering flight is particularly energetically costly (Suarez et al., 1990) and, consequently, represents a compelling subject with which to investigate the tradeoffs that occur in the energetic and aerodynamic demands of flight across large elevational gradients. In this study, we explored how *Patagona gigas peruviana,* a population of Giant Hummingbirds inhabiting mid to high elevations (~2500 - 4500 m), alters major flight parameters when confronted by the distinct air densities and oxygen partial pressures at low and high elevation sites. We analysed wingbeat kinematics, measured metabolic power input and

calculated mechanical power output of Giant Hummingbirds at a high elevation site (3800 m; site of capture) and again at sea level (~50 m).

To avoid introducing bias in our analyses, we initially tested for sexual differences in morphology and aerodynamic flight parameters at both elevations. Morphological variables did not differ between males and females except for body mass, in which males were significantly larger than females. However, sample sizes were inadequate for comparisons of morphological variables; they were only measured in two females and two males, except for body mass where sample size was larger (nine males and six females). Moreover, we found no significant differences in wingbeat kinematics, metabolic power input and mechanical power output between males and females at both elevations. To properly quantify the effect of gender on flight performance further tests on a larger sample size need to be done. Given the challenges of living at high elevations and the cost imposed by the Giant Hummingbird's body mass, it would be interesting to investigate how females, oblige to gain mass before the breeding season (e.g. egg synthesis), cope with these challenges during that time, assuming that they do not migrate to lower elevations.

As seen in previous studies of smaller hummingbird species (Chai and Dudley, 1995; Altshuler and Dudley, 2003; Altshuler et al., 2010), stroke amplitude, mechanical power output and metabolic power input all increased significantly with elevation in the Giant Hummingbird. However, contrary to what has previously been shown, this large hummingbird also increased wingbeat frequency significantly (~6%). In fact, it increased by about the same percentage as the increase in stroke amplitude (~8%). This may be explained by the high stroke amplitude characteristic of this species, which is ~152 degrees at 50 m, compared to ~148 degrees and 154 degrees at ~1800 m.a.s.l in *Selasphorus platycercus* and *Selasphorus rufus* respectively (Altshuler and Dudley, 2003). Because Giant Hummingbirds have extremely high wing stroke amplitudes even at low elevations, they have less compensatory excess capacity than do smaller hummingbird species. Consequently, we conclude that Giant Hummingbirds must modulate wing stroke amplitude and wingbeat frequency in order to adjust for reduced density air.

Patagona gigas peruviana inhabits high elevations and may already be acclimated or adapted to this challenging environment. In this study, instead of subjecting individuals to extreme conditions (as previous studies have done; see Altshuler and Dudley, 2003), they were transported to lower elevations where it should be easier to balance aerodynamic forces and energetic demands. Had we subjected *P. gigas peruviana* to a more extreme environmental conditions, we may have found results similar to those found in smaller species, where wing stroke amplitude would mainly increase, while leaving wingbeat frequency unchanged.

Assuming that there are strong correlations between the supply of oxygen and the demand of the working tissues during hovering flight (Bishop and Butler, 1995; Fernández (Chapter 2); Fernández et al., *submitted*), we expected to find an increase in metabolic power similar to the increase in mechanical power at high elevations relative to sea level. However, we found that at high elevations metabolic power increased by 33% while mechanical power only increased by 12% relative to that at sea level (~50 m), suggesting an enhanced metabolic cost of hovering flight at high elevations. At present it is unclear why metabolic costs would increase at a substantially greater rate than mechanical power output. Perhaps, exploring how the Giant Hummingbird modulates detailed wingbeat kinematics (e.g. wing rotation, angle of attack, torsion along the wing) might help to explain the unexpected lack of correlation betwen mechanical and metabolic power.

Patagona gigas gigas is a subspecies that inhabits mainly low elevations up to 2500 m. Studying this group in comparison to *P. gigas peruviana* could also elucidate the mechanical and physiological mechanisms that have enabled the Giant Hummingbird to adapt to extremely different environmental conditions in spite of the high cost. Mass-specific induced power requirements in hovering flight are proportional to the square root of wing loading (Ellington 1984c). Thus, the populations that inhabit high elevations should have larger wings than lowland populations. This relationship has indeed been shown in previous studies looking at the intra-and interspecific comparisons in hummingbird species inhabiting different elevations (Feisinger et al., 1979; Altshuler and Dudley, 2002; Altshuler and Dudley, 2003; Altshuler et al., 2004). Preliminary data on the morphology of the lowland, *P. gigas gigas*, showed a significant decrease in wing length compared to the high elevation population measured in this study (124.5 mm versus 134.3 mm, respectively; Wilcoxon test, $P < 0.05$; Fernández and Dudley, unpublished data). Moreover, these comparisons may shed some light on the evolutionary trade-offs and constraints that have shaped the history and the evolutionary potential of hummingbird species.

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Tables and Figures

Table 3.1. Morphological variables for *P. gigas peruviana* separated by gender, for use in aerodynamic calculations. Values are mean ± s.e, and sample sizes (n) are in parenthesis. Body mass (*Mb*), wing length (*R*), total wing area (*S*) (i.e., area for both wings), aspect ratio (*AR*), wing loading (p_w).

Gender	*Mb* (g)	*R* (mm)	*S* (mm^2)	*AR*	p_w (N/m^2)
Female	19.6 ± .5 (6)	139.7 ± 3.1 (2)	8615.6 ± 535.9 (2)	8.3 ± .1 (2)	21.5 ± .1 (2)
Male	22.6 ± .5 (9)	143.0 ± .2 (2)	9016.7 ± 296.9 (2)	9.1 ± .3 (2)	24.0 ± .1 (2)

Table 3.2. Wingbeat kinematics values and power requirements during hovering flight at two different elevations. Values are mean ± s.e., sample size (N), *t*-value (t) and p-value (*P*). Wingbeat frequency (*n*), stroke amplitude (Φ), hovering mass-specific metabolic rate (HMR/*M*), and mechanical power output assuming perfect elastic storage (P_{per}).

	Elevation				
	Low (sea-level)	High (3800 m)	N	*t*	*P*
n (Hz)	13.0 ± .1	13.9 ± .2	6	4.8	0.005
Φ (degrees)	152.0 ± 1.5	160.4 ± 2.0	6	3.9	0.011
HMR/*M* (mlO_2/gh)	23.7 ± 1.1	31.7 ± 1.6	5	-3.5	0.025
P_{per} (W/kg)	22.1 ± .7	24.8 ± .6	4	-6.2	0.008

Figure Legends

Figure 3.1: Pooled data from males (N= 4) and females (N= 2) expressed as mean values ± s.e at low and high elevations (50 and 3800 m, respectively) (a) wingbeat frequency (Hz) and (b) wing stroke amplitude (degrees).

Figure 3.2: Pooled data from males (N= 2) and females (N= 2) expressed as mean values ± s.e at low and high elevations (50 and 3800 m, respectively) of mechanical power assuming perfect elastic storage, Pper (W/kg).

Figure 3.3: Pooled data from males (N= 4) and females (N= 2) expressed as mean values ± s.e at low and high elevations (50 and 3800 m, respectively) of mass-specific hovering metabolic rate ($mlO_2\ g^{-1}\ h^{-1}$).

Figure 3.1a

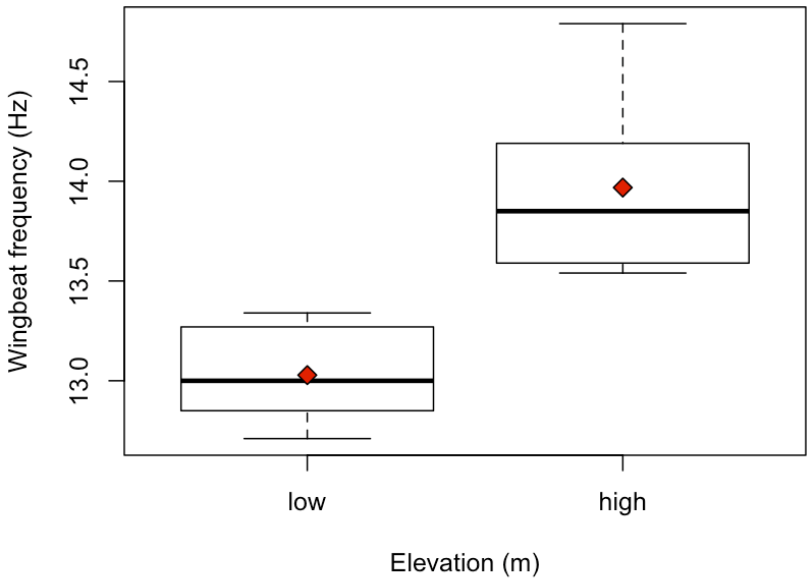


Fig 3.1b

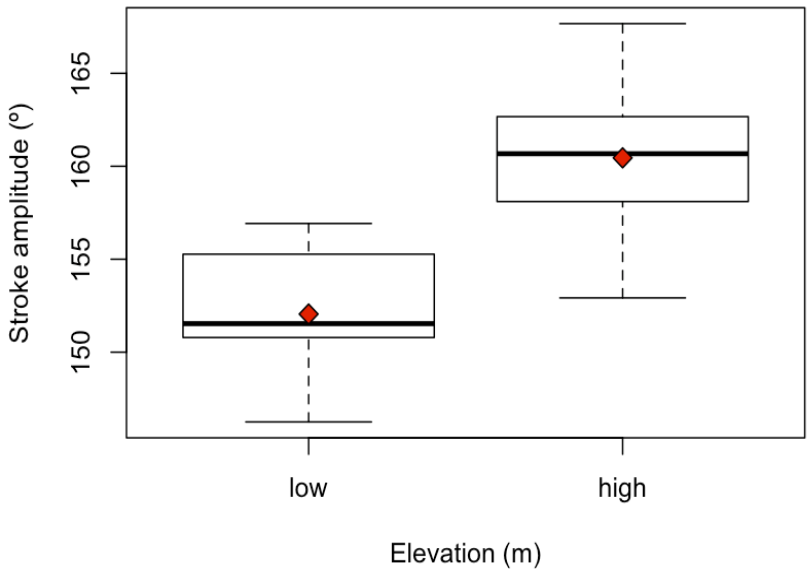


Fig. 3.2

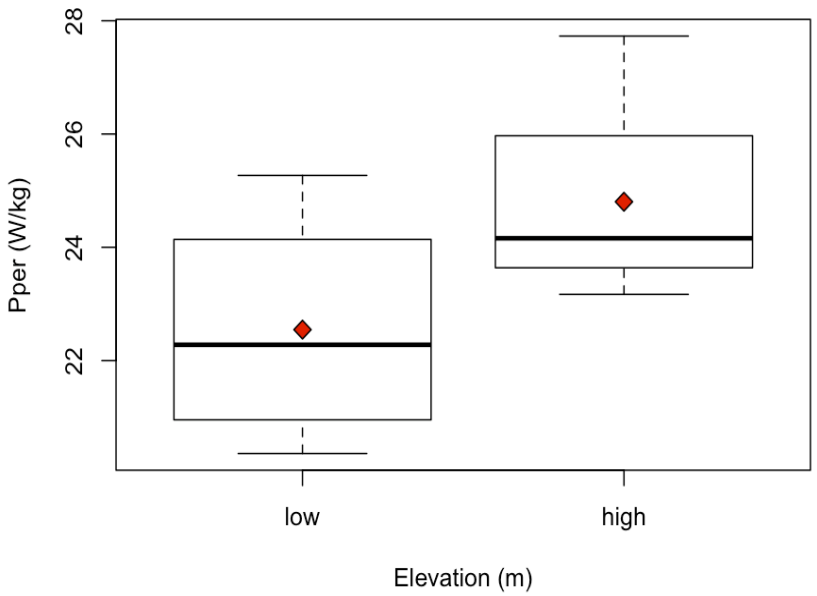
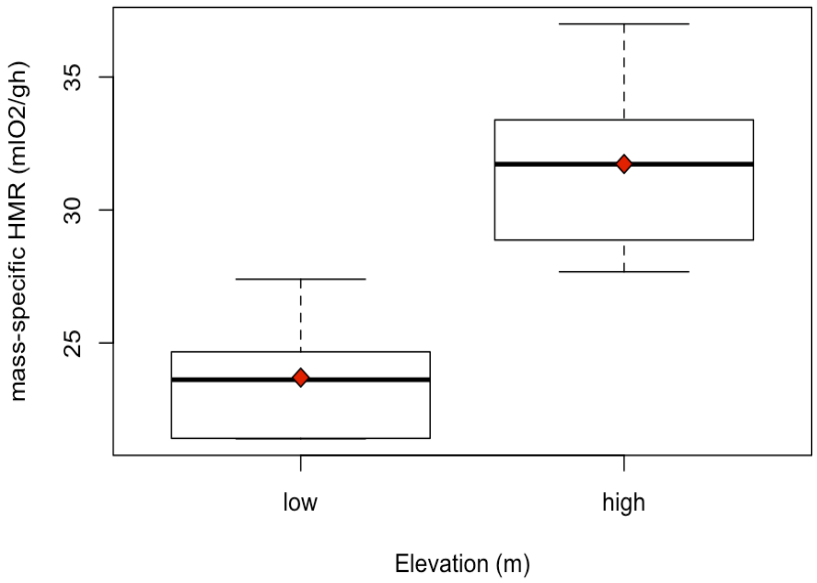


Fig. 3.3



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