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 $M^{0.78}$ (M is mass), 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 g. 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 regulator concentrations) dominates as the proximate explanation for the interspecific variation in flux. On the other hand, we found no evidence of hierarchical regulation of flux, which results from variation in V_{max} and is based on variation in enzyme concentration [E]. The evolutionary conservation of pathways of energy metabolism suggests that "one size fits all" among hummingbirds.

Résumé : Les colibris (famille des Trochilidés) sont parmi des plus petits vertébrés endothermes, et présentent une physiologie extrême chez les oiseaux. Le vol sur place, l'une des formes de locomotion les plus coûteuses, est une caractéristique qui leur est propre. Sachant que le coût métabolique du vol sur place (HMR) chez les colibris est fonction allométrique de $M^{0,78}$ (M est la masse), nous avons testé l'hypothèse selon laquelle HMR pourrait être corrélé aux changements d'activité maximale (V_{max}) d'enzymes jouant un rôle clé dans les processus d'oxydation du glucose et des acides gras dans les muscles du vol chez quatre espèces de colibris dont la masse varie de 4 à 20 g. En parallèle, nous avons également estimé les flux métaboliques à partir de données de respirométrie mesurées durant le vol sur place. Contrairement à notre hypothèse, aucune corrélation entre V_{max} et les flux métaboliques n'a été trouvée, en particulier pour les réactions métaboliques impliquant l'hexokinase et la carnitine palmityl transférase. Dans le cadre d'une analyse de régulation hiérarchique, ces résultats indiquent que la régulation métabolique (provenant de changements de substrat, produit ou effecteur allostérique) explique le mieux les variations interspécifiques de flux métabolique. D'un autre côté, nous n'avons mis en évidence aucun signe de régulation hiérarchique du flux métabolique, c'est-à-dire aucun changement de V_{max} dû à un changement de concentration en enzymes [E], provenant d'une régulation de l'expression génique. La conservation au fil de l'évolution de ces processus métaboliques suggère l'existence d'une « taille métabolique unique » chez les colibris.

Introduction

Hummingbirds (Trochilidae) are characterized 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* (Lembeye, 1850)) and 22 g (Giant Hummingbird, *Patagona gigas* (Vieillot, 1824)) (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 1986; Suarez 1992; Clark and Dudley 2009) and fast forward flight (Berger 1985; Clark and Dudley 2009, 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 $\dot{V}o_2$ (rate of oxygen consumption) and $\dot{V}co_2$ (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 and flux rates through pathways of substrate oxidation (Brand 2005; Suarez et al. 1990).

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Previous studies on Rufous (*Selasphorus rufus* (Gmelin, 1788)), Anna's (*Calypte anna* (Lesson, 1829)), and Broad-tailed (*Selasphorus platycercus* (Swainson, 1827)) hummingbirds 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, 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, 1990). However, such biochemical data have been obtained only from *S. rufus*. 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 rate (HMR) scale allometrically as $M^{0.78}$ (where M is mass) among species ranging from 3 to 20 g (Fernández 2010; Fernández et al. 2011). In addition, not all hummingbird species engage in hover-feeding behaviour; some species perch while drinking floral nectar (Carpenter 1976). The reduced energetic cost of foraging might be reflected in the evolutionary design of pathways of energy metabolism in the flight muscles of such species.

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, which is 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 regulator 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 HMR. 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 (Oasis Hummingbird (*Rhodopis vesper* (Lesson, 1829)), Green-backed Firecrown (*Sephanoides sephaniodes* (Lesson, 1827)), Andean Hillstar (*Oreotrochilus estella* (Orbigny and Lafresnaye, 1838)), and *P. gigas*), ranging in body mass from 4 to 20 g. 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 *O. estella*. This species inhabits exclusively high elevations (above ~3000 m above sea level (asl)) 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 2010; Fernández et al. 2011). 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), and 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 and July 2002. *Patagona gigas* ($n = 4$), *O. estella* ($n = 4$), and *R. vesper* ($n = 4$) were captured in northern Chile, Chusmiza (19°40′S, 69°10′W; ~3583 m asl), and *S. sephaniodes* ($n = 3$) were captured in central Chile (33°17′S, 71°11′W; ~600 m asl). Hummingbirds were transported to the Pontificia Universidad Católica de Chile in Santiago (33°27′S, 70°40′W; ~520 m asl), 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. Enzyme activities remained stable for at least 6 years when stored at –80 °C, based on a comparison of enzyme activity between our flight muscle samples for *P. gigas* collected in 2002 and flight muscle samples (kindly provided by the Museum of Vertebrate Zoology at the University of California Berkeley) from three *P. gigas* collected in Peru in June–August 2006. Except for PFK, which was not detectable in any of the tissues, mean V_{max} values for the rest of the enzymes were not significantly different when comparing samples collected at different times, during 2002 and 2006 (Wilcoxon test: all $P > 0.07$).

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 mmol·L^{-1} HEPES (pH 7.0), 50 mmol·L^{-1} imidazole (pH 7.1), 50 mmol·L^{-1} Tris-Cl (pH 7.2) or 50 mmol·L^{-1} sodium phosphate (pH 7.4) with 2 mmol·L^{-1} EDTA, 0.5% (*v*/*v*) Triton X-100, and 5 mmol·L^{-1} 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 No. MS-50 (Heat Systems Ultrasonics Inc., Farmingdale, New York, USA) and then centrifuged for 4 min at 10 000*g* at 4 °C using an IEC Micromax refrigerated microcentrifuge (Needham Heights, Massachusetts, USA). Supernatant fractions in microcentrifuge tubes were kept in ice until assays were completed.

Maximum enzyme activities

We measured V_{max} of seven enzymes involved in the glu-

cose and fatty acid oxidation pathways. Four enzymes participate in carbohydrate metabolism: (1) glycogen phosphorylase (GP; EC 2.4.1.1) catalyzes the degradation of glycogen; (2) hexokinase (HK; EC 2.7.1.1) catalyzes glucose phosphorylation and entry into the glycolytic pathway; and (3) 6-phosphofructokinase (PFK; EC 2.7.1.11) is an allosteric enzyme that may play a role in the control of glycolysis; (4) L-lactate dehydrogenase (LDH; EC 1.1.2.3) catalyzes a near-equilibrium reaction that under certain conditions may lead to net lactate production. Two mitochondrial enzymes involved in the fatty acid oxidation pathway were measured: (1) 3-hydroxyacyl-CoA dehydrogenase (HOAD; EC 1.1.1.35) is an enzyme in the β-oxidation pathway and (2) carnitine *O*-palmitoyltransferase (CPT; EC 2.3.1.21) 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. Although we refer to the activity measured simply as CPT in this paper, we note that it is likely that the assay measured CPT-2 and not CPT-1 because of the use of Triton X-100 in the homogenization buffer (see Suarez et al. 2009). We measured citrate synthase (CS; EC 2.3.3.1), which catalyzes the first reaction in the Krebs cycle and serves as a mitochondrial marker (Moyes 2003). Assay buffers used in this study were the same as reported by Suarez et al. (2009). Briefly, we used 50 mmol·L^{-1} Tris-Cl (pH 8.35) for PFK, CPT, and CS; 50 mmol·L^{-1} imidazol (pH 7.25) for HOAD; 50 mmol·L^{-1} sodium phosphate (pH 7.4) for GP; and 50 mmol·L^{-1} HEPES (pH 7.15) for HK and LDH. 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 Grant Instruments Ltd., 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, when 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 Wilcoxon 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. Correlation between mean flux rate in vivo (Fernández 2010; Fernández et al. 2011) and mean flux capacity in vitro measured in this study was tested using a Spearman's correlation test. All statistical analyses were performed using R (R Development Core Team 2010).

Results

Mean values for V_{max} in four trochilid species, ranging in mean body mass from 4.7 to 16.3 g, are given in Table 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, $P > 0.06$; for detailed statistics see Table 2). However, we found a significant difference in mean values of CS (Kruskal–Wallis test; $\chi^2_{[3]} = 9.58$, $P = 0.022$) and HOAD (Kruskal–Wallis test; $\chi^2_{[3]} = 9.33$, $P = 0.025$). A posteriori Wilcoxon tests showed that CS activity was higher (203.3 μmol·min^{-1}·(g wet mass)$^{-1}$) in the smallest hummingbird (i.e., *R. vesper*; 4.7 g) and significantly different from the other species (Wilcoxon test; *S. sephaniodes*: $P = 0.05$; *O. estella*: $P = 0.02$; *P. gigas*: $P = 0.02$). The lowest CS activity (148.1 μmol·min^{-1}·(g wet mass)$^{-1}$) was found in the medium-sized species (i.e., *S. sephaniodes*; 5.5 g), which had the greater variance compared with the other species. The difference found in CS between species was not dependent upon body mass (CS ∝ $M^{-0.07}$; $r^2 = 0.02$, $P = 0.44$). HOAD activity was higher in *S. sephaniodes* (343.3 μmol·min^{-1}·(g wet mass)$^{-1}$). However, a posteriori Wilcoxon tests showed that HOAD activity in *S. sephaniodes* was not significantly different compared with the other species (Wilcoxon test; *R. vesper*: $P = 0.06$; *O. estella*: $P = 0.11$; *P. gigas*: $P = 0.06$). The only significant difference found in HOAD activity was between *O. estella* (7.6 g) and *P. gigas* (16.3 g) (Wilcoxon test; $P = 0.03$). Also, the variation in HOAD between species was not accounted for by body mass (HOAD ∝ $M^{-0.16}$; $r^2 = 0.23$, $P = 0.07$). In general, therefore, V_{max} values were largely independent of body mass.

ATP turnover and metabolic flux rates during hovering were estimated using respirometric data (Fernández 2010; Fernández et al. 2011) (Table 3). Muscle mass scales isometrically with body mass in hummingbirds; on average, flight muscle mass consists of 26% of total body mass ($n = 23$ species (Altshuler and Dudley 2002); $n = 4$ species (M.J. Fernández, unpublished data)), where 90% or more of whole-body rates of oxygen consumption occurs (Suarez 1992; Taylor 1987). Assuming that only glucose is oxidized 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 ± SE), respectively. To sustain the above rates of ATP turnover, hummingbirds require the oxidation of glucose at a rate between 10.4 and 19.0 μmol·g^{-1}·min^{-1}. When fasted hummingbirds oxidize fatty acids (e.g., palmitate) during hovering, the rates of oxidation would be between 2.0 and 3.7 μmol·g^{-1}·min^{-1}, with a P/O ratio of 2.09 (Brand 2005). Mean values for glucose and palmitate oxidation rates during hovering flight are given in Table 4. For both substrates, the oxidation rates differ significantly between species (Kruskal–Wallis test: $\chi^2_{[3]} = 10.99$, $P = 0.01$). Specifically, *S. sephaniodes* require oxidation rates 44% higher on both substrates (glucose and palmitate) compared with *O. estella* (Table 4). 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 oxidizing pathways (flux rates ∝ $M^{-0.19}$; $r^2 = 0.33$, $P = 0.02$).

Within species, the mean body mass of the individuals used for respirometry (Fernández 2010; Fernández et al. 2011) was not significantly different from those used for the enzyme V_{max} measurements (Wilcoxon test; *S. sephaniodes*: $P = 0.88$; *R. vesper*: $P = 0.08$; *O. estella*: $P = 0.85$; *P. gigas*: $P = 0.63$). 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 sig-

Table 1. Body mass (g) and maximum enzyme activities (V_{max}; $\mu mol \cdot min^{-1} \cdot (g\ wet\ mass)^{-1}$) in flight muscles of four hummingbird species.

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

Note: Common names are Oasis Hummingbird (*Rhodopis vesper*), Green-backed Firecrown (*Sephanoides sephaniodes*), Andean Hillstar (*Oreotrochilus estella*), and Giant Hummingbird (*Patagona gigas*).

Table 2. Kruskal–Wallis test showing the difference in maximum enzyme activities (V_{max}) in flight muscles between four hummingbird species (Oasis Hummingbird (*Rhodopis vesper*), Green-backed Firecrown (*Sephanoides sephaniodes*), Andean Hillstar (*Oreotrochilus estella*), and Giant Hummingbird (*Patagona gigas*)).

Enzyme	χ^2	df	*P*
Hexokinase (HK)	6.22	3	0.101
L-Lactate dehydrogenase (LDH)	6.28	3	0.100
Carnitine *O*-palmitoyltransferase (CPT)	2.65	3	0.448
Citrate synthase (CS)	9.58	3	0.022*
Hydroxyacyl-CoA dehydrogenase (HOAD)	9.33	3	0.025*
Glycogen phosphorylase (GP)	7.45	3	0.068

Note: *, $P < 0.05$.

nificantly correlated (Spearman's rank correlation; HK: $P = 0.417$; CPT: $P = 0.333$). If it is assumed that only glucose is oxidized during hovering, then 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 oxidized, the v/V_{max} at the CPT step ranges from 0.44 to 0.84 (Table 4).

Discussion

Design of pathways of energy metabolism

The high V_{max} values for HK, CPT, and CS 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, 1990; Welch et al. 2006), allowing hummingbirds to switch between these fuels, depending on prandial state and flight behaviour. Qualitatively, the four Chilean species are identical to *S. rufus* (Suarez et al. 1986, 1990) in terms of the overall design of pathways of energy metabolism. Also in common with *S. rufus*, HK and CPT in the Chilean species operate at high fractional velocities (v/V_{max}) during hovering flight when either glucose or fatty acid is oxidized (Table 4). 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 2010; Fernández et al. 2011) 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 HMR 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. However, body mass did not explain the variation in the V_{max} values of CS and HOAD. 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. Even *O. estella* (the high-elevation, perching species) displayed V_{max} values well within the range of values found across species in this study. However, given the small number of species included here, it is not possible to disentangle the influences of feeding behaviour and altitude.

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). HK 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, they 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

Table 3. Body mass (g) and mass-specific metabolic rate during hovering flight (HMR; mL $O_2 \cdot g^{-1} \cdot h^{-1}$) for four hummingbird species (*n* is sample size).

	R. vesper		*S. sephaniodes*		*O. estella*		*P. gigas*	
	Mean ± SE	*n*	Mean ± SE	*n*	Mean ± SE	*n*	Mean ± SE	*n*
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	

Note: Respirometric data was adapted from Fernández et al. (2011). Briefly, $\dot{V}o_2$ were obtained from a hummingbird feeder altered to function as a respirometry mask pulling air at 1000 $mL \cdot min^{-1}$. The 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 HMR 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. Common names are Oasis Hummingbird (*Rhodopis vesper*), Green-backed Firecrown (*Sephanoides sephaniodes*), Andean Hillstar (*Oreotrochilus estella*), and Giant Hummingbird (*Patagona gigas*).

Table 4. Required substrate oxidation rates ($\mu mol \cdot g^{-1} \cdot min^{-1}$), maximum possible rates of flux and fractional velocities (v/V_{max}) through hexokinase (HK) and carnitine palmitoyltransferase (CPT) in flight muscles of four hummingbird species during hovering flight.

	Oxidation rate					
	Required		Maximum possible		v/V_{max}	
	Mean ± SE	*n*	Mean ± SE	*n*	HK	CPT
Glucose oxidation						
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	
Palmitate oxidation						
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

Note: In the calculations, it was assumed that 90% of $\dot{V}o_2$ was due to flight muscles (Suarez, 1992) and that flight muscle mass was 26% of total body mass (Altshuler and Dudley 2002; M.J. Fernández, unpublished data). Stoichiometries of glucose and palmitate oxidation were from Brand (2005). Common names are Oasis Hummingbird (*Rhodopis vesper*), Green-backed Firecrown (*Sephanoides sephaniodes*), Andean Hillstar (*Oreotrochilus estella*), and Giant Hummingbird (*Patagona gigas*).

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 HK and CPT steps are independent of body mass, whereas 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 parameters that modulate muscle power (Ellington 1984). Altshuler and Dudley (2003) showed that wingbeat frequency decreases as $M^{-0.21}$, whereas stroke amplitude is independent of body mass (Altshuler et al. 2010). If we make the assumption that muscle stress and strain scale isometrically across hummingbird species, then we would expect muscle volume-specific (or mass-specific) power output (Pennycuick and Rezende 1984) to scale negatively with mass. Because 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, whereas 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*]. This may result 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 regulators. 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 and 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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