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MITOCHONDRIAL OXIDATIVE CAPACITIES: Does functional capacity match maximum physiological demand?

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by

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ABSTRACT

MITOCHONDRIAL OXIDATIVE CAPACITIES: Does functional capacity match maximum physiological demand?

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The oxygen-dependent processes of ATP synthesis in mitochondria is same in all mammals and birds (endothermic animals). However, some endothermic species have higher maximum O_2 consumption rate per unit mass (VO_2max/Mb) than others during aerobic exercise. It is unclear whether the mitochondria of these endothermic species achieving higher VO_2max/Mb have inherently higher biochemical capacities for respiration than mitochondria from other species. To understand the relation between functional capacities (biochemical capacity) and maximum physiological requirements, the respiration rates of flight muscle mitochondria of hovering *C. anna* (in vivo mVO_2) and the maximum oxygen consumption rate of isolated mitochondria (in vitro mVO_2) were compared. To measure whole body VO_2max of *C. anna*, *C. anna* hovered in heliox (21% oxygen and 79% helium gas mixture). In vivo mVO_2 of *C. anna* flight muscles calculated using whole body VO_2max was 10.5 ml O_2/min x ml on average. Since in vitro mVO_2 values are substrate-dependent, in vitro mVO_2 was measured using different combinations of substrates: ones that donate electrons to the electron transport chain via NADH and those that donate electrons via both NADH and $FADH_2$ (Gnaiger, 2009). The NADH and $FADH_2$ –linked substrate combination

elicited the highest in vitro mVO_2 which was 6.8 ml O2/min × ml on average. The in vitro mVO_2 measurement methods used in this study brought a gap between in vivo and in vitro rates closer than the previous study (Suarez et al., 1990); however, biochemical capacity was still 17.3 % lower than the maximum physiological rate. *C. anna* flight muscle cytochrome aa3 content measured using spectrophotometric method was 83.88 n mol/g muscle. The electron flux through cytochrome aa3 calculated using cytochrome aa3 content and VO_2 of *C. anna* hovering in heliox at 86.7 electrons/second. This electron flux rate is only slightly higher than the rate of rat heart, but cytochrome aa3 content per gram muscle is about 6 times higher in hummingbird flight muscle than the rat heart muscle. This indicates the level of cytochrome aa3 expression and the physiological requirements are matched to maximum requirements in both hummingbird pectoral and rat cardiac muscles.

The relationship between mitochondrial respiratory capacity *in vitro* and maximum respiration rate *in vivo* in hummingbird flight muscles

Introduction

When animals engage in aerobic exercise, the demand for energy by their locomotory muscles increases as does the rate of respiration (VO_2). Rates of processes that hydrolyze adenosine-5'-triphosphate (ATP) increase and VO_2 increases because mitochondria of locomotory muscle cells need to re-synthesize ATP using oxygen-dependent processes. The process of ATP synthesis in mitochondria of all mammals and birds (endothermic animals) are the same; however, the maximum oxygen consumption rate per unit mass (VO_2max/Mb) during aerobic exercise varies among endothermic animals. Hovering hummingbirds have the highest VO_2/Mb among endothermic animals. During maximum aerobic exercise (VO_2max), more than 90 % of whole body O_2 consumption and CO_2 production are due to mitochondria of locomotory muscle cells (Taylor, 1987). It is known that hummingbirds possess relatively more flight muscle mass and that these flight muscles possess more mitochondria than other species of birds (Suarez, 1992). In this study, the following questions were asked: 1) what is the maximum rate at which muscle mitochondria function in hovering hummingbirds *in vivo*? 2) how does the maximum rate achieved in the living animal *in vivo* compare with the maximum capacity for respiration of isolated mitochondria *in vitro*? 3) do hummingbird flight muscle mitochondria have inherently higher capacities for respiration than

mitochondria from other species? To answer these questions, Anna's hummingbird (*Calypte anna*) flight muscles were used. Hummingbird flight muscles are homogeneous and consist of only one type of fiber (Welch Jr and Altshuler, 2009). Thus, whole body VO_2max/Mb can be used to estimate the maximum metabolic rate of a single muscle fiber type. Whole body VO_2max of *C. anna* was measured using a mixture of 79% helium and 21% oxygen (heliox) (Chai and Dudley, 1996). Hummingbirds hovering at normal air density typically do not exhibit VO_2max. The density of heliox is lower than normal air and hummingbirds need to increase wing stroke frequency and amplitude in heliox above that in normal air and this causes them to achieve VO_2max. In this present study, VO_2max was estimated using linear regression analysis. Using this estimated VO_2max, the maximum physiological rate of respiration of these mitochondria *in vivo* (in vivo mVO_2) was estimated.

The maximum oxygen consumption rate of mitochondria isolated (in vitro mVO_2) from *C. anna* flight muscle *in vitro* was also measured since measurement of in vitro mVO_2 allows comparison of the capacity of these mitochondria for respiration with the maximum in vivo mVO_2 estimated during hovering at VO_2max. Values of in vitro mVO_2 are substrate-dependent and measurements of in vitro mVO_2 when physiological substrates (pyruvate + malate) are provided have invariably underestimated maximal respiratory capacities because of loss of Krebs cycle intermediates into the assay medium (Gnaiger 2009). As a result of incomplete Krebs cycle function, insufficient succinate is made. This causes little or no electron flow from complex II to coenzyme Q in the electron transport system (ETS) (Gnaiger 2009). Therefore, in this study, to determine the substrate combination which elicits the maximum in vitro mVO_2, I measured in vitro mVO_2 of *C. anna* flight muscle mitochondria using different combinations of substrates: substrate combinations that provide

electron flow from complex I to coenzyme Q and substrate combinations that provide electron flow from both complex I and complex II to coenzyme Q. The maximum in vitro mVO_2 of *C. anna* flight muscle was compared with the maximum mitochondria *in vivo* rate estimated during hovering at VO_2max. The comparison of the respiratory capacity of isolated mitochondria with respiration rates estimated from VO_2max/Mb in animals hovering in helium-oxygen mixtures would reveal whether biochemical capacities closely match or exceed maximum physiological requirements during hovering flight.

Electron flow through the transport system (ETS) of mitochondria is a process essential to the synthesis of ATP. However, details concerning the structure of ETS are still under debate. Cytochrome aa3, which catalyzes the terminal step of the mitochondrial ETS, is an enzyme essential to the synthesis of ATP. Thus, cytochrome aa3 levels in hummingbird flight muscles were analyzed quantitatively using a spectrophotometric method by Dr. Robert Balaban's laboratory at the NIH. The content of mitochondrial cytochrome aa3 and the VO_2max of hummingbirds hovering in heliox was used to estimate the turnover rate of cytochrome aa3. Thus, in addition to providing insights into the relationship between biochemical capacities and maximum physiological requirements, this study provides some insights into the function of the ETS of mitochondria in hummingbird flight muscles, the most metabolically active, aerobic muscles known among vertebrate animals.

Material and Methods

Male Anna's hummingbirds (*Calypte anna*) were captured, maintained and studied with approval from California Department of Fish and Wildlife, U.S. Fish and Wildlife Service and the Institutional Animal Care and Use Committees (IACUC) at the University of California (Riverside and Santa Barbara).

Because of the size limitation of hummingbird flight muscles, it was not feasible to analyze the cytochrome aa3 content of flight muscle mitochondria and measure the mitochondrial oxygen consumption rates on the flight muscles from the same individuals. Birds used to measure the maximum oxygen consumption rates (VO_2max) of hovering hummingbirds and to analyze the cytochrome aa3 content of flight muscle mitochondria and birds used to measure the mitochondrial oxygen consumption rates (in vitro mVO_2) were different.

To measure VO_2max of hovering hummingbirds and to analyze the cytochrome aa3 content of flight muscle mitochondria quantitatively, four male Anna's hummingbirds were captured with a hall trap (100 cm diameter x 100 cm height) on the premises of UC Riverside in July and August, 2010. Captured hummingbirds were individually housed in 90 cm x 100 cm x 90 cm cages in a temperature- and humidity-controlled vivarium. These hummingbirds were given free access to commercially-available hummingbird food (Nektar-Plus, NEKTON USA, Clearwater, FL).

To measure in vitro mVO_2 of flight muscle mitochondria, four male Anna's hummingbirds were captured on the premises of UC Santa Barbara in 2012 using the same methods mentioned above. These captured birds were transferred to the laboratory for the measurement of the respiration rate of flight muscle mitochondria.

Respirometry

Respirometry measurements of hovering hummingbirds were carried out in an acrylic chamber (1 m x 1m x 1m) using mask respirometry as described in Suarez et al. (1990), Welch (2011), Welch et al. (2006), Welch et al. (2007), and Welch and Suarez (2007) with some modification. A feeder was suspended from the ceiling of the acrylic chamber. The

feeder was set near one corner of the chamber with a two-wing distance from the walls. Diagonally across from the feeder, a perch was set. Air was drawn through the mask, through a Tygon tube connected to a CA-2A carbon dioxide analyzer (Sable Systems International, Las Vegas, NV) and into a FOXBOX oxygen analyzer (Sable Systems International, Las Vegas, NV) with a built-in pump. The flow rate of air was set at 1200 ± 200 ml/s, which was calibrated using a Singer DTM-115 dry gas flow meter located downstream of the FOXBOX. Drierite (8 mesh indicating, W.A. Hammond Drierite, Xenia, OH) in a tube (3/4" outside diameter x 8" overall length) placed upstream of the CO_2 analyzer and Ascarite (8-20 mesh, Thomas® Ascarite II CO_2 Absorbent, Thomas Scientific, Swedesboro, NJ) in a tube located upstream of the O_2 analyzer were used to scrub H_2O and CO_2 respectively from respired air. While hummingbirds are feeding, hummingbirds insert their heads in and remove their heads from the feeder repeatedly. To measure the duration of the one feeding and the number of head insertion and removal movement during one feeding event, the mask was equipped with an infrared emitter and detector system (motion detector). Analog data from O_2 and CO_2 analyzers, and the motion detector were converted into digital signals using a UI-2 Data acquisition interface analog-to-digital converter (Sable Systems International, Las, Vegas, NV) and sent to a computer where these data were recorded using Expedata Build, version 1.2.5 data acquisition and analysis program software (Sable Systems International, Las, Vegas, NV).

The density of air in the chamber was adjusted using the method described by Dudley (1995), Altshuler et al. (2001), Altshuler and Dudley (2003) and Chai et al. (1999) with some modification. Ambient air density (1.2 kg/m^3 at sea level) in the chamber was lowered by replacing it with the mixture of 79 % helium (0.166 kg/m^3) and 21 % oxygen (1.325 kg/m^3)

gas (Praxair, Inc., Danbury, CT). A MFC-2 Mass Flow Controller gas mixer (Sable Systems International, Las, Vegas, NV) was used to mix oxygen and helium (heliox) and control the rate of heliox flow into the chamber at a rate of 10L/min. A small fan installed in the chamber was used to ensure complete mixing. Change in air density was monitored acoustically as described in Dudley (1995), Altshuler et al. (2001), Altshuler and Dudley (2003) and Chai et al. (1999). Acoustic tones were recorded using a Raven Lite 1.0 sound analysis program (Bioacoustics Research Program, Cornell Lab of Ornithology, Ithaca, NY). Analysis of the air density from acoustic tone was carried out using a MATLAB analysis program (version R2009b, MathWorks, Inc. Natick, MA).

The oxygen analyzer was calibrated with ambient air drawn through the mask in the absence of a hummingbird. The carbon dioxide analyzer was calibrated with nitrogen gas (CO_2 free) and 0.5 % CO_2 gas (Praxair, Danbury, CT).

In preliminary experiments, the lowest air density at which a hummingbird failed to hover was determined for each hummingbird. VO_2 was measured while air density was gradually decreased down to the air density just above the lowest air density. A hummingbird was allowed access to the feeder every 15 min. Body mass was manually recorded before feeding and after feeding during experiment when a hummingbird sat on a perch which was set on a digital balance. Body mass of each pre-feed and post-feed bird was averaged and this average body mass was used to calculate a mass specific VO_2.

In order to determine VO_2, the traces of STP-corrected oxygen depletion were first corrected using baseline values. These baseline-corrected data were then converted to ml of gas according to Lighton (2008). Rates of oxygen consumption were determined by dividing the volume of respired gasses by the feeding duration. To estimate reliable metabolic rates,

feeding bouts were used for analysis when these two conditions were met: if a feeding bout was more than 4 sec and if the average length of an individual feeding event (head insertion and removal) within a feeding bout was more than 1 sec. If the average length of individual feeding events within a bout was less than1 sec, that entire bout was excluded from the analysis (Welch 2011, Welch and Suarez 200).

Cytochrome aa3 content in flight muscle

The quantity of cytochrome aa3 in flight muscles of the hummingbirds used for the respiratory measurement in Heliox were analyzed quantitatively using a spectrophotometric method at the National Institute of Health. The method for the measurement of cytochrome aa3 in flight muscle was according to Balaban et al. (1996) with some modification. Minced frozen muscle tissues was placed in 9 volumes of HEPE buffer (280 mM sucrose, 10 mM HEPES, 1 mM EDTA, 1 mM EGTA, pH 7.1 @ 4 °C) and homogenized at 40% power for 15 sec on ice using a Virtishear (Virtis, Gardiner, NY). Cyt a, a3 content was determined by adding 250 µl homogenate to 750 µl Triton+Pi and centrifuging at 16,000 g for 5 min. The absorbance of the supernatant was recorded from 550 – 650 nm (oxidized) and then again after the addition of 10 mM cyanide and 10 mM ascorbate (reduced). Cyt a, a3 content was calculated using the optical absorbance difference between the oxidized and reduced spectra with a millimolar extinction coefficient of 10.8. Using the cytochrome aa3 content and the VO_2 of hummingbirds hovering in heliox, the electron turnover rate through cytochrome aa3 was calculated.

Mitochondrial oxygen consumption rate

Mitochondria were isolated from flight muscles of Anna's hummingbirds using the method described by Suarez et al. (1986) with some modification. All chemicals used in this

study were from Sigma Aldrich and Fisher Scientific. Birds were euthanized by decapitation. Then, both pectoralis and supracoracoideus muscles were quickly dissected out with a surgical scalpel blade. A small portion of flight muscles was cut out, frozen immediately with dry ice and kept in -80 C° freezer. After quickly washing flight muscles with 175mM KCl solution three times, muscles were minced with a pair of surgical scissors in an ice cold isolation buffer consisting of 10 mM Tris-Cl (pH 7.4), 70 mM sucrose, 210 mM mannitol, and 1 mM ethyleneglycolbis(P-aminoethyl ether)-N,N'-tetraacetic acid (EGTA). 1mg/10ml protease Type VIII from *Bacillus licheniformis* was added to the minced muscle, which was then homogenized. Homogenization was carried out with a loose-fitting Teflon pestle driven by a T-Line laboratory stirrer (Talboys engineering Corp. South Montrose, PA) in Potter-Elvehjem homogenization vessel. The speed of the stirrer was 60% of its maximum and the minced muscle suspension was stroked 8 – 10 times. After homogenization, 10 ml of isolation buffer containing 3mg/ml of bovine serum albumin (BSA, fatty acid free, less than 98%) was immediately added to the homogenate. All isolation processes were conducted at 0-4 C°.

The homogenate was spun for 5 min at 50 g in a Jouan MR14.11 refrigerated centrifuge at 4 C°. The isolation buffer containing 3mg/ml BSA was then added to the supernatant containing mitochondria. Mitochondria were sedimented as a light brown pellet by centrifuging the supernatant at 3500 g. The pellet was washed twice, the first time by resuspension in 3 ml of fresh isolation buffer and centrifugation at 3000 g for 5 min. The second wash involved resuspension in isolation 3 ml buffer and centrifugation at 2500 g for 5 min. The pellet from the last spin was resuspended with 0.5 µl of the isolation buffer containing 3mg/ml of BSA and kept in ice.

The measurement of mitochondrial respiration rates were carried out in a Gilson 1.7 ml water-jacketed glass cell as described in Suarez et al. (1986) and Suarez et al. (1991) with some modification. The assay buffer consisted of 10 mM mannitol, 110 mM sucrose, 10 mM potassium phosphate ; 10 mM Tris-Cl (pH 7.4), 2.5 mM magnesium chloride and 3 mg/ml BSA. Rates of O_2 consumption were monitored with a Clark-type O_2 electrode (5331A, YSI, Yellow Springs, OH) connected to an amplifier (YSI 5300A Biological Oxygen Monitor, YSI, Yellow Springs, OH). A Teflon-covered stir bar installed in the cell and a magnetic stirrer were used to ensure constant mixing during assays. The amplifier was connected to a computer via a CB405 connector box (Sable Systems International, Las, Vegas, NV) and analog output from the amplifier was converted to digital recording using a Datacan V5.0 data acquisition and analysis program (Sable Systems International, Las Vegas, NV). Incubation temperature was maintained at 39 °C by circulating 39 °C water using a RM6 water bath circulator (Lauda-Brinkmann, Delran, NJ). For calibration, air-saturated assay buffer at 39 °C was assumed to contain 397 nmol of oxygen/ml (Reynafarje et al. 1985).

Mitochondrial respiration rates (in vitro mVO_2) were measured using four different substrate combinations: 1) pyruvate + malate, 2) pyruvate + malate + glutamate, 3) pyruvate + malate + succinate, and 4) pyruvate + malate + glutamate + succinate as in vitro mVO_2 values are substrate-dependent (Gnaiger 2009). Respiratory control (RCR) and ADP/O ratio were calculated as in Estabrook (1967). Respiratory states are defined according to Chance and Williams (1956).

Mitochondrial protein measurement

Mitochondrial protein was measured according to Gornall et al. (1949) by using 10% deoxycholate to solubilize membrane proteins and bovine serum albumin (BSA, fatty acid

free, less than 98%) as the standard. Protein concentration was determined by measuring an absorbance set at 540 nm using a Pharmacia LKB Novaspec II UV/VIS Spectrophotometer (Amersham Pharmacia Biotech, Piscataway, NJ).

Citrate synthase assay for muscle tissue and mitochondria

The activity of citrate synthase (marker enzyme for mitochondria) from flight muscles and isolated mitochondria was measured as described in Suarez et al. (1986) and Suarez et al. (1991) with some modification. Citrate synthase (CS) activity in mitochondrial suspension and muscle samples were used to normalize in vivo and in vitro respiration rates.

Frozen muscle sample was minced in 9 volumes of the homogenization buffer consisting of 20 mM Tris-Cl (pH 7.6), 0.5 % (v/v) Triton X-100, 2 mM ethylenediaminetetraacetic acid (EDTA) and 5 mM 2-Mercaptoethanol (BME). Muscle suspension was homogenized 4 times for 10 seconds at 30 seconds intervals with a Pro 200 homogenizer (Pro Scientific Inc. Oxford, CT) at 50% of its maximum speed. Muscle homogenate was sonicated 4 times for 10 seconds each with 30 second intervals between each sonication with a sonicator (Microson ultra sonic cell distuptor, Ultrasonics-Heat System, Farmingdale, NY) at 30% of its maximum output power. All processes were carried out between 0-4 C°. After sonication, homogenate was kept in an ice bath.

Mitochondrial suspension was resuspended with 4 volumes of a cold 20 mM Tris-Cl buffer (pH 7.6) containing 20 mM Tris-Cl (pH 7.6), 0.5 % (v/v) Triton X-100, and 2 mM EDTA. The suspension was sonicated 4 times for 10 seconds at 30 seconds intervals with a sonicator at 30% of its maximum output power. Homogenate was spun at 12000 g with an IEC Micromax OM 3590 refrigerated microcentrifuge (IEC, Needham Heights, MA) for 4 minutes. All processes were carried out between 0-4 C°. Supernatant was kept in an ice bath.

CS from muscle tissues and from isolated mitochondria was assayed in 50 mM Tris-Cl (pH 8.0), 0.5 mM oxaloacetate (omit for control), 0.3 mM acetyl coenzyme A (Acetyl CoA), 0.1 mM 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB). CS assay was carried out at 39 °C.

The activity of CS was determined by measuring a change in absorbance set at 412 nm using a Pharmacia LKB Novaspec II UV/VIS Spectrophotometer (Amersham Pharmacia Biotech, Piscataway, NJ) connected to a computer. All data obtained from the spectrophotometer were recorded with a Datacan V5.0 data acquisition and analysis program (Sable Systems International, Las Vegas, NV). A millimolar extinction coefficient of 13.6 was used to calculate CS activity.

Calculation and Statistics

VO_2 at sea level, VO_2 in heliox (lower density) and VO_2 at the lowest density which birds was able to hover were presented for each bird (Table 1). VO_2 at sea level was an average VO_2 for that bird measured at sea level. Linear regression analysis was used to estimate the VO_2max at the lowest density from all VO_2 for that bird measured at sea level and at the lower density. Two-tailed Mann-Whitney U test were used for all data analyses.

Results

Maximum oxygen consumption rate

The O_2 consumption rates of hovering hummingbird were measured at sea level and at lower density using heliox. The mass-specific VO_2 of hovering hummingbirds at sea level and the estimated mass-specific VO_2 at the lowest density are given in Table 1. The lowest density at which each hummingbird was able to sustain hovering more than 4 seconds varied from 0.73 to 0.49 kg/m^3.

VO_2 of hummingbirds hovering at normal air density was 37.39±6.12 ml O_2/g x h on average, which is close to values obtained in previous studies (Bartholomew and Lighton, 1986; Welch et al, 2007). As the air density lowed, VO_2 of all hummingbirds increased. The estimated VO_2 was 46.24±7.15 ml/g × h in average, which is 22.7 % higher than at normal sea level air density. Although all four birds had lower values for VO_2 at sea level than at the lowest air density, the number of birds precludes the possibility of statistically significant results (P=0.2).

Cytochrome aa3 content and electron turnover rate

Cytochrome aa3 content and electron turnover rate are summarized in Table 2. The electron turnover rate was calculated using cytochrome aa3 content and oxygen consumption rate according to Suarez et al. (1999). Electron turnover rate was calculated using the estimated VO_2. Electron turnover rate was increased 24.5±11.0 % during hovering at the lowest air density relative to hovering at normal air density.

Mitochondrial oxygen consumption rates

The respiratory control ratios (RCR) and ADP/O which indicate the functional integrity of mitochondria and mitochondrial oxygen consumption (in vitro mVO_2) are summarized in Table 3. RCR for pyruvate + malate as substrates was 5.0±0.62 on average, which is close to the value obtained in previous studies (Suarez et al. 1986). ADP/O ratio for pyruvate + malate was 2.21±0.04, which is close to the value estimated by Brand (2005). CS activity in mitochondrial suspension was 2.93±0.06 units/mg mitochondrial protein in average. CS activity measured using muscle tissue was 472.1±60.7 units/g muscle on average and close to the value reported in Suarez et al. (1990).

The value for in vitro mVO_2 using pyruvate + malate was 176.5±4.99 n mol/mg mitochondrial protein × min, which is higher than the in vitro mVO_2 using the same substrates obtained in previous studies (Suarez et al, 1991). There is no difference in in vitro mVO_2 between pyruvate + malate and pyruvate + malate + glutamate (P = 0.114). When only pyruvate + glutamate were used as substrate, no respiration was observed (data not shown). In vitro mVO_2 was 1.5 fold and 2.5 fold higher when pyruvate + malate + succinate and pyruvate + malate + glutamate + succinate respectively (P=0.021 for both) were used as substrates than when pyruvate + malate were used as substrates.

The comparison of in vitro and in vivo oxygen consumption rates

In vitro and in vivo oxygen consumption rates are presented in Table 4. The purpose of this study was to compare the maximum oxygen consumption rate of mitochondria and whole animal oxygen consumption rate. In vivo oxygen consumption rates were estimated using oxygen consumption rates during hovering at the lowest density. Oxygen consumption rates were calculated using the highest mVO_2 (Pyruvate + Malate + Glutamate + Succinate as substrates). However, birds used to measure VO_2 and birds used to measure mVO_2 were different.

Both in vitro and in vivo oxygen consumption rates were expressed in ml O_2/mg mitochondrial protein. Although in vitro VO_2 increased by using Pyruvate + Malate + Glutamate + Succinate, in vitro VO_2 were still 17.3±7.95 % lower than the in vivo VO_2 at normal air sea level density (statistically not different) and 34.0±7.71 % lower than in vivo VO_2 at the lower air density (marginally significant).

Discussion

Oxygen consumption rate (VO_2) vs. Mitochondrial respiration rate (mVO_2)

In the present study, I attempted to understand the biochemistry of flight muscle mitochondria of hummingbirds which have the highest oxygen consumption rate during aerobic exercise among vertebrates. The approach involved comparing the mitochondrial respiration rates (mVO_2) estimated from VO_2max/Mb in animals hovering in normal-density air and in helium-oxygen mixtures with the respiratory capacity of isolated mitochondria.

By lowering the air density using heliox, hummingbirds respired at the rate of 46 ml/g x hr; this is about 1.2 times more than the VO_2 at sea level. By titrating with pyruvate + malate + glutamate + succinate to reconstruct the tricarboxylic acid cycle (TCA) (Gnaiger, 2009), the mVO_2 measured, 440.2 n mol O_2 / mg protein x min, is 2.5 times and 2.8 greater than the mVO_2 with pyruvate + malate measured in this study and mVO_2 reported by Suarez et al., 1986, respectively. The gap between in vivo mitochondrial respiration rate at sea level and biochemical capacity (in vitro mVO_2) is 17.3 %; the difference between them is not significantly different ($P>=0.05$). The gap between physiological capacity at lower air density and biochemical capacity is 34 %; the difference between them is marginally significant ($P<0.05$). The methods used in this study to measure in vitro mVO_2 results in a closer match between in vivo and in vitro rates than the previous study (Suarez et al., 1990).

Heliox was also used to induce VO_2max of Anna's hummingbird; however, VO_2 at the lowest density is about 1.2 times lower than the value of 52 ml / g x hr for ruby throated hummingbirds measured in heliox by Chai and Dudley (1996) and the value of 53.1 ml/g x hr for male Anna's hummingbirds measured at sea level by Epting (1980). The physiological condition of Anna's hummingbirds in this study may have been different, rendering them unable to perform as well as birds in these other studies. For example, the body mass of Bird 4 whose VO_2 were 35.9 ml / g x hr at sea level and 44.4 ml / g x hr at the lowest density was

4.22 g in captivity, but it became 3.9 g at the end of the heliox study. Another possibility is measurement error. For example, Bird 2 whose VO_2 was the lowest of all, moved its head frequently, 25 times in a 13 second feeding event or 5 times in a 2 second feeding event. Bird 2 spent the least time at the feeder. The average feeding time was 6.7 seconds. The ratio of the total duration for one feeding even to the total number of head insertion and removal movement occurred during one feeding event was 1.2 on average which is the smallest of all. It is possible that this feeding behavior caused some air sample escape from the mask and might have caused underestimation of VO_2.

mVO_2 was measured in vitro by reconstituting the TCA cycle; however, hummingbird flight muscle mitochondria respired at the rate of 440.2 n mol O_2 / mg protein × min which is lower than the respiration rate of 672.4 n mol O_2/mg mitochondrial protein × min and 787.9 n mol O_2/mg mitochondrial protein × min by the mitochondria with pyruvate + malate + glutamate in pectoralis muscle of English sparrow (*Passer domesticus*) and in hindlimb muscle of rat (*Rattus norvegicus*), respectively, measured by Kuzmiak et al. (2012). It is possible that the concentration of ADP and inorganic phosphate added to measure the mVO_2 in this study was not high enough to stimulate the respiration of mitochondria fully (Gnaiger 2009). Also, it was observed that mitochondrial respiration was further stimulated (result not shown here) by titrating cytochrome c at state 4. It is possible that the outer membranes of some isolated mitochondria were broken and some cytochrome c was lost.

Respiration of mitochondria in hummingbird flight muscle was not observed using glutamate alone or the mixture of glutamate + malate as substrate, while adding glutamate to a substrate mix of pyruvate + malate did not increase the mVO_2 further. This is interesting because glutamate alone is a substrate for mitochondrial respiration in human skeletal muscle

(Gnaiger, 2008) and pigeon pectoralis (Rasmussen and Rasmussen, 2004). The mVO_2 with a combination of malate + glutamate is about 1.2-fold higher than the mVO_2 with a combination of pyruvate + malate as substrate in pigeon pectoralis (Rasmussen and Rasmussen, 2004), in sparrow pectoralis and is about 1.1-fold higher in rat hindlimb (Kuzmiak et al., 2012). In sparrow pectoralis and in rat hindlimb, a mixture of pyruvate + malate + glutamate is oxidized at a rate 1.3-fold higher and 1.2-fold higher respectively than the mVO_2 with a combination of pyruvate + malate as substrate (Kuzmiak et al., 2012). Glutamate dehydrogenase activity was not detected (Suarez et al., 1986) in hummingbird flight muscle. However, the rate of mitochondrial respiration was further increased when glutamate was provided as a substrate in combination with pyruvate + malate + succinate. A possible explanation for this is the high activity of glutamate-oxaloacetate transaminase (GOT) (Suarez et al., 1986). GOT catalyzes the reaction

oxaloacetate + glutamate ↔ aspartate + α-ketoglutarate

and is found in both cytoplasmic and mitochondrial compartments. In my in vitro experiments, mitochondrial GOT could have catalyzed the conversion of added glutamate (plus oxaloacetate, made through Krebs cycle reactions from added succinate) to α-ketoglutarate (plus aspartate). This mechanism would allow glutamate to further increase mVO_2 even in the absence of glutamate dehydrogenase, but only when there is oxaloacetate to react with, which comes from added succinate.

Cytochrome aa3 content and electron turnover rate

Cytochrome aa3 is the final enzyme in the ETS that carries out the final transfer of the electrons to oxygen and is essential to the synthesis of ATP. In the present study, cytochrome aa3 in hummingbird flight muscles was analyzed quantitatively and its electron

turnover rate was calculated using cytochrome aa3 content and the VO_2 of hovering hummingbirds to gain insights into the rate of electron flux through the mitochondrial ETS of hummingbird flight muscle.

C. anna flight muscle cytochrome aa3 (cyt aa3) content was 83.88 n mol/g muscle and the electron flows through cyt aa3 were 69.7 electrons/second and 86.7 electrons/second when *C. anna* was hovering in normal air and in heliox, respectively. When it is compared with honeybees whose estimated mass-specific VO_2 is 109 ml/g/hr, the electron turnover rate in flight muscles of hummingbird hovering in heliox is almost 1/10 of the turnover rate (799 electrons/second) in honeybee thorax (Suarez et al., 1999). The turnover rate in heliox is only slightly higher than the rate of rat heart (53.6 electrons/second) (Table 5), but the cyt aa3 content per gram muscle is about 6 times higher in hummingbird flight muscle than the rat heart muscle. This suggests that the level of expression of cytochrome aa3 is roughly proportional to the requirements of both hummingbird pectoral and rat cardiac muscles.

In the present study, I attempted to determine the relationships between mitochondrial capacities in vitro and maximum physiological requirements in vivo as well as to ask whether hummingbird flight muscle mitochondria have inherently higher capacities for respiration than mitochondria from other species. The gap between physiological capacity and biochemical capacity became smaller than the one reported by Suarez et al. (1990); however, physiological rate is still higher than biochemical capacity since it is probable that mitochondria in the present study were not fully activated.

Using VO_2 and mitochondrial protein content, in vivo mitochondrial oxygen consumption rate (in vivo mVO_2) can be estimated. VO_2 of hummingbird hovering in heliox in this study was 2.47 ml O_2/g muscle × min and mitochondrial protein content found in this

study was 162.23 mg mitochondrial protein/g muscle. These give an in vivo mitochondrial respiration rate of 679.69 n mol O_2/mg mitochondrial protein × min, which is similar to in vitro mVO_2 of sparrow flight muscle (672.4 n mol O_2/mg mitochondrial protein × min) and rat hindlimb (787.9 n mol O_2/mg mitochondrial protein × min). However, hummingbirds hovering in heliox in the present study may not have respired at a maximum rate as mentioned above. It is possible that hummingbird flight muscle mitochondria would respire at a higher rate than sparrow flight muscle mitochondria. The estimated value for in vivo mVO_2 of hummingbird flight muscle is higher than in vitro mVO_2 for other species. However, it is possible that in vitro mVO_2 of other species may also be underestimated since it is reported that rat hindlimb mitochondria respired at the rate of 787.9 n mol O_2/mg mitochondrial protein × min (Kuzmiak et al., 2012). As Gnaiger (2009) pointed out in his review paper, only NADH-related substrates such as pyruvate, malate and glutamate have been used to measure in vitro mVO_2 of other species in many other studies. As the present study showed, that there is no difference in in vitro mVO_2 between the mixture of pyruvate + malate and the mixture of pyruvate + malate + glutamate. However, when succinate was added to the mixture of pyruvate + malate + glutamate, this gave the highest in vitro mVO_2 values. It is not easy to do preliminary studies to establish the optimum substrate combination and concentration when the number of individuals can be used is limited and the sample sizes for measurement are small. It is beneficial to conduct preliminary studies using the different mixture of substrates, especially the mixture of pyruvate + malate + glutamate + succinate.

There is no statistically significant difference between in vitro and in vivo rates obtained at sea level, and there is only a marginally significant difference between rates in

vitro and in vivo at lower density. In the present study, biochemical rate (capacity) more closely matches physiological rate than in the previous study by Suarez et al. (1991).

Table 1. The comparison of oxygen consumption rates of hummingbirds hovering at sea level and at lower air densities.

Bird ID	VO_2 at sea level	RQ for sea level	Lowest air density hummingbird was able to hover	Estimated VO_2max at the lowest air density
1	44.95±2.06	0.89±0.06	0.64	55.73348
2	30.39±0.50	0.97±0.03	0.49	38.51513
3	39.48±1.41	0.97±0.08	0.73	46.36317
4	35.93±5.36	0.96±0.07	0.62	44.35352
Avg.	37.69±6.12	0.94±0.04	0.62±0.10	46.24±7.15

1. VO_2 is expressed in ml/g x h

2. Air density is expressed in kg/m^3

3. VO_2max at the lowest air density is estimated using a linear regression equation.

Table 2. Hummingbird flight muscle cytochrome aa3 content and an electron flux through cytochrome aa3.

Bird ID	cyt aa3	Turnover rate in vivo at sea level	Turnover rate in vivo at the lowest air density	% increase
1	94.10	81.7	102.1	25.0
2	76.04	61.4	81.9	33.2
3	86.13	65.3	71.1	8.9
4	79.24	70.2	91.7	30.6
Avg.	83.88±8.01	69.7±8.79	86.7±13.26	24.5±11.0

1. Cytochrome aa3 is expressed in n mol/g wet weight.

2. Turnover rate is expressed in e^- flux/ sec x n mol cyt aa

3. The electron turnover rate is calculated using cytochrome aa3 content and oxygen consumption rate according to Suarez et al (1999).

Table 3: The comparison of substrate dependent mitochondrial respiration, coupling and respiratory control ratios (RCRs) in mitochondria from hummingbird flight muscles.

	Pyruvate+Malate	**Pyruvate+Malate +Glutamate**	**Pyruvate+Malate +Succinate**	**Pyruvate+Malate +Glutamate+Succinate**
mitochondrial respiration rates	176.45±4.99	165.55±12.98	255.5±24.79	440.2±58.25
P values Pyruvate+Malate		0.114	0.021#	0.021#
Pyruvate+Malate +Glutamate			0.021#	0.021#
Pyruvate+Malate +Succinate				0.021#
RCR	5.0±0.62	4.6±0.62	1.7±0.26	2.8±0.47
RCR (oligomycin)	12.0±9.06	7.5±5.46	-	-
ADP/O ratio	2.21±0.04	2.21±0.12	1.47±0.18	1.51±0.24

n = 4

1. Mitochondrial respiration rate is expressed in n mol O_2/mg mito protein x min
2. #: 2-tailed Mann-Whitney U nonparametric test gives P = 0.021 for these due to the small sample size.
3. - : Since state 4 respiration did not change after addition of oligomycin, RCR (oligomycin) for these are the same as RCR.
4. Concentration of substrates used to measure mVO_2 is 5 mM pyruvate, 0.1 mM malate, 5 mM glutamate, 5 mM succinate and 277.6 nM ADP. Oligomycin was used to inhibit respiration.

Table 4: The comparison of in vitro and in vivo oxygen consumption rates.

	In vivo		In vitro	% difference	
Bird ID	**VO_2 at sea level**	**VO_2 @ lowest air density**	**mVO_2**	**Sea level VO_2 vs. mVO_2**	**Lowest air density VO_2 vs. mVO_2**
1	11.2	14.1	8.0	-28.7	-43.5
2	7.0	9.1	5.8	-16.6	-36.2
3	7.3	8.8	6.5	-11.0	-25.4
4	7.9	10.0	6.9	-12.9	-31.0
Avg.	8.3±1.93	10.5±2.47	6.8±0.90	-17.3±7.95	-34.0±7.71
P values				0.2	0.0286

1. Both VO_2 and mVO_2 are expressed in ml/min x ml mitochondria.
2. The calculation for in vivo and in vitro oxygen consumption rates were according Suarez et al (1999).

Table 5: Comparison of cytochrome aa3 content and electron turnover rate of different species

	Cyt aa3/wet wt (n mol/g)	Turnover rate (e^-/second)
Hummingbird (sea level)	83.9	69.7
Hummingbird (lower density)		86.7
Honeybee thorax (a	21.25	799
Rat heart (b	14.25	53.6

a) Suarez et al., 1999
b) Nishiki et al., 1978 referred in Suarez et al., 1999

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