© 2016. Published by The Company of Biologists Ltd | Journal of Experimental Biology (2016) 219, 3719-3728 doi:10.1242/jeb.142711

RESEARCH ARTICLE

Mitochondrial physiology in the skeletal and cardiac muscles is altered in torrent ducks, *Merganetta armata*, from high altitudes in the Andes

Neal J. Dawson[1,2,‡], Catherine M. Ivy[1], Luis Alza[2,3,4], Rebecca Cheek[3], Julia M. York[5], Beverly Chua[5], William K. Milsom[5], Kevin G. McCracken[2,3,*] and Graham R. Scott[1,*]

ABSTRACT

Torrent ducks inhabit fast-flowing rivers in the Andes from sea level to altitudes up to 4500 m. We examined the mitochondrial physiology that facilitates performance over this altitudinal cline by comparing the respiratory capacities of permeabilized fibers, the activities of 16 key metabolic enzymes and the myoglobin content in muscles between high- and low-altitude populations of this species. Mitochondrial respiratory capacities (assessed using substrates of mitochondrial complexes I, II and/or IV) were higher in highland ducks in the gastrocnemius muscle – the primary muscle used to support swimming and diving – but were similar between populations in the pectoralis muscle and the left ventricle. The heightened respiratory capacity in the gastrocnemius of highland ducks was associated with elevated activities of cytochrome oxidase, phosphofructokinase, pyruvate kinase and malate dehydrogenase (MDH). Although respiratory capacities were similar between populations in the other muscles, highland ducks had elevated activities of ATP synthase, lactate dehydrogenase, MDH, hydroxyacyl CoA dehydrogenase and creatine kinase in the left ventricle, and elevated MDH activity and myoglobin content in the pectoralis. Thus, although there was a significant increase in the oxidative capacity of the gastrocnemius in highland ducks, which correlates with improved performance at high altitudes, the variation in metabolic enzyme activities in other muscles not correlated to respiratory capacity, such as the consistent upregulation of MDH activity, may serve other functions that contribute to success at high altitudes.

KEY WORDS: High altitude, Mitochondrial respiration, Energy metabolism, Myoglobin, Muscle energetics

INTRODUCTION

The torrent duck (*Merganetta armata*, Gould 1842) inhabits fast-flowing rivers over a wide elevational range in the Andes (Johnsgard, 1972; Carboneras, 1992). They are remarkably strong swimmers and are able to dive and fly at altitudes from sea level to greater than 4500 m, where barometric pressure and oxygen availability are low and P_{O_2} is less than 60% of that at sea level (Swan, 1970; Johnsgard, 1972). There is very little known about the physiological strategies used by swimming and diving bird species to overcome the challenges of high-altitude environments. Previous studies of torrent ducks suggest that they, like other high-altitude birds, have evolved morphological enhancements to support flight at altitude, including smaller body sizes and larger wingspans (Feinsinger et al., 1979; Lee et al., 2008; Gutiérrez-Pinto et al., 2014). However, it is unknown whether highland populations of torrent ducks possess physiological or enzymatic specializations that help match O_2 supply and demand while swimming and diving under hypoxic conditions.

Torrent ducks live year-round in some of the most powerful and fast-flowing rivers, resulting from the run-off of ice-capped mountains to create waterfalls and rivers, surrounded by rugged and steep mountain slopes, in the Andes (Koepcke, 1970; Todd, 1996). Due to the harsh nature of their environment, torrent ducks are considered precocial, and have a long incubation period to ensure proper development for young ducklings to be able to swim and survive in the violent waters of their environment (Johnsgard, 1968; Johnson and Moffett, 1972; Todd, 1996). Torrent ducks are very strong swimmers and divers, relying predominantly on swimming for transportation, with little emphasis on flying. Torrent ducks are notoriously cautious, and typically swim with the majority of their bodies submerged to avoid detection (Johnsgard, 1968, 1992; Todd, 1996). Torrent ducks are territorial, vocal and very aggressive in warding off invaders, and a pair of torrent ducks will occupy a length of river with little movement in their lifetime (Baldassarre and Bolen, 1994; Todd, 1996).

Birds that migrate long distances at high altitudes have been shown to have an elevated capacity for transporting O_2 to the flight muscle during hypoxia. Putative adaptations seem to have arisen across the O_2 transport pathway that enhance breathing, pulmonary gas exchange, hemoglobin–O_2 affinity, capillarity of the flight muscle and heart, and oxidative capacity of the flight muscle (Petschow et al., 1977; Weibel, 1984; Jessen et al., 1991; Zhang et al., 1996; Scott and Milsom, 2006, 2007; McCracken et al., 2009a,b; Scott et al., 2008, 2009a,b, 2015a,b; Natarajan et al., 2015). Much less is known about whether high-altitude species have similarly altered the phenotypes of other muscles to help support other forms of locomotion and/or shivering in the hypoxic and cold environment at high elevation. Goslings of the bar-headed goose (*Anser indicus*) – a species that migrates across the Himalayas, and for which some populations hatch and spend their summers on the Tibetan Plateau – have higher capillarity in the gastrocnemius muscle than hatchling Canadian geese (*Branta canadensis*) even

[1]Department of Biology, McMaster University, 1280 Main Street West, Hamilton, Ontario L8S4K1, Canada. [2]Department of Biology and Department of Marine Biology and Ecology, Rosenstiel School of Marine and Atmospheric Sciences, University of Miami, Coral Gables, FL 33146, USA. [3]Institute of Arctic Biology and University of Alaska Museum, University of Alaska Fairbanks, Fairbanks, AK 99775, USA. [4]Centro de Ornitología y Biodiversidad - CORBIDI, Lima 33, Peru. [5]Department of Zoology, University of British Columbia, Vancouver, British Columbia V6T1Z4, Canada.
*These authors contributed equally to this work

‡Author for correspondence (neal.dawson@gmail.com)

N.J.D., 0000-0001-5389-8692

Received 5 May 2016; Accepted 7 September 2016

Journal of Experimental Biology

Journal of Experimental Biology (2016) 219, 3719-3728 doi:10.1242/jeb.142711

when both species are raised and compared in normoxia (Hawkes et al., 2013; Snyder et al., 1984). The extent to which this occurs and is perhaps enhanced in birds that use the leg muscles for active locomotion at altitude is unknown.

Our objective was to examine and compare the mitochondrial physiology and metabolic properties of flight muscle (pectoralis), swimming muscle (gastrocnemius) and cardiac muscle (left ventricle) between high- and low-altitude populations of torrent ducks in their native environment. There are three subspecies of *M. armata*: *M. a. colombiana* from Venezuela, Colombia and Ecuador; *M. a. armata* from Argentina and Chile; and *M. a. leucogenis* from Peru and Bolivia (Carboneras, 1992). We studied populations of *M. a. leucogenis* across altitudes in two adjacent valleys in the department of Lima in Peru (approximately 70 km northeast of Lima city). We measured mitochondrial respiratory capacities using permeabilized muscle fibers (measured in the field at the native altitude), as well as the maximal activities of 16 key metabolic enzymes and myoglobin (Mb) content in muscle tissue.

MATERIALS AND METHODS

Animals

Male torrent ducks were captured at high altitudes (3000–4086 m; P_{O_2}=15.05–13.167 kPa) on the Chancay River valley near Vichaycocha, or at low altitudes (1092–1665 m; P_{O_2}=18.81–17.556 kPa) on the Chillón River valley in Santa Rosa de Quives. Ducks were captured by herding them upriver into mist nets, and were then transported in pet carriers to each respective field lab at high altitude (3547 m: −11.1414482, −76.6230056) or low-altitude (1172 m: −11.6688815, −76.7902587). Ducks were allowed to recover with unlimited access to water for 12–18 h and were then euthanized by cervical dislocation. Samples of left ventricle, gastrocnemius and pectoralis muscles were quickly dissected for use in mitochondrial respiration experiments or frozen in liquid N_2 and stored at −80°C for enzyme analysis (see below). Samples of each tissue were taken at a site of intermediate depth from the outer muscle surface. Samples were collected and imported to Canada with appropriate permits: Permiso Para Fauna y Flora Silvestre of the Peruvian ministerio de agricultura y riego (nos 190-2015-SERFOR/0002445-SERFOR) and Scientific Collection permit from the Canadian Wildlife Service (no. POS 369). All procedures were carried out in accordance with guidelines set out by the Canadian Council on Animal Care, and were approved by institutional animal care committees.

Permeabilized muscle fiber experiments

Small samples (~50 mg) of left ventricle, gastrocnemius and pectoralis muscles were transferred to ice-cold relaxing and preservation buffer (20 mmol l^{-1} imidazole, 2.77 mmol l^{-1} CaK_2EGTA, 7.23 mmol l^{-1} K_2EGTA, 6.56 mmol l^{-1} $MgCl_2$, 20 mmol l^{-1} taurine, 0.5 mmol l^{-1} DTT, 50 mmol l^{-1} potassium-methane sulfonate, 5.8 mmol l^{-1} Na_2ATP and 15 mmol l^{-1} creatine phosphate, pH 7.1). Fibers were mechanically separated in relaxing and preservation buffer using dissecting probes with the assistance of watchmakers' glasses, and were then chemically permeabilized for 30 min in the same buffer containing 50 µg ml^{-1} saponin. Fibers were then rinsed three times for 10 min in respiration buffer [20 mmol l^{-1} Hepes, 0.5 mmol l^{-1} EGTA, 3 mmol l^{-1} $MgCl_2$, 60 mmol l^{-1} potassium lactobionate, 20 mmol l^{-1} taurine, 10 mmol l^{-1} KH_2PO_4 and 110 mmol l^{-1} sucrose, and 1 mg ml^{-1} fatty-acid-free bovine serum albumin (BSA); pH 7.1] to wash out endogenous metabolites.

In situ mitochondrial respiration was measured in 2 ml of respiration solution under continuous stirring in a glass, water-jacketed respiration chamber. P_{O_2} was measured continuously using a FireStingO_2 fiber-optic oxygen meter and OROBIO oxygen probe (Pyroscience, Aachen, Germany), and was recorded using Pyro Oxygen Logger software (Pyroscience). Temperature was measured using a FireSting temperature probe and was held at 40.75°C (maintained to within ±0.02°C) using a water heater/recirculator. Fibers (2.1–5.3 mg dry weight) were allowed to rest for 5 min after being transferred to the chamber, and then malate (2 mmol l^{-1}) followed by pyruvate (5 mmol l^{-1}) were added to stimulate leak-state respiration. ADP (2.5 mmol l^{-1}) was added to elicit maximal ADP-stimulated respiration via complex I, reflecting the mitochondrial capacity for pyruvate oxidation. Respiration was then measured after each addition of glutamate (5 mmol l^{-1}) and succinate (25 mmol l^{-1}). The resulting maximal ADP-stimulated respiration after succinate addition represents the full capacity for oxidative phosphorylation via complexes I+II with convergent electron inputs to coenzyme Q. Cytochrome *c* (10 µmol l^{-1}) was then added to assess the viability of the preparations (increases in respiration via exogenous cytochrome *c* provides an index for outer-mitochondrial-membrane integrity; preparations that had increases of 5% or more in respiration after addition of cytochrome *c* were deemed unfit for analysis; only one run showed a significant cytochrome *c* effect). Finally, ascorbate (0.5 mmol l^{-1}) followed by *N*,*N*,*N*,*N*-tetramethyl-*p*-phenylenediamine (TMPD; 0.5 mmol l^{-1}) was used to maximally stimulate complex IV. Respiration rates were measured for at least 3 min at each state in order to reach a steady-state respiration value, and rates are expressed relative to the wet weight of fibers. Because there can be significant barriers to O_2 diffusion between the bulk respiration solution and the interior of the fiber bundle lattice, O_2 concentration was maintained above 160 µmol l^{-1} throughout the experiment by bubbling pure O_2 into the respiration chamber via PE10 tubing as needed (preliminary experiments found that maximal respiration was not O_2 limited above 160 µmol l^{-1} O_2, in agreement with previous observations by Scott et al., 2009a,b). Biochemicals were obtained from Sigma-Aldrich (Oakville, ON, Canada) unless otherwise stated.

Enzyme activity assays on homogenized muscle

The maximal activities of 16 enzymes were assayed at avian body temperature (41°C) for left ventricle, pectoralis and gastrocnemius muscles. Samples were homogenized in 10 volumes of ice-cold homogenizing buffer A [100 mmol l^{-1} KH_2PO_4 buffer, pH 7.2, containing 1 mmol l^{-1} EGTA, 1 mmol l^{-1} EDTA and 1 mmol l^{-1} phenylmethylsulfonyl fluoride (PMSF)]. Homogenates were then centrifuged at 1000 ***g*** at 4°C and the supernatant collected for use in enzyme assays. Measured activities were assayed in triplicate. Enzyme activity was determined as the difference between the rate measured using all assay components and the background reaction rate [rate in the presence of inhibitor ('+') or without key substrate ('−')]. Measurements were carried out in 100 mmol l^{-1} KH_2PO_4 (pH 7.2) under the following assay conditions.

For the electron transport chain: (1) complex I [CI; ε=6.22 (mmol l^{-1})$^{-1}$ cm^{-1} at 340 nm]: 0.01 mmol l^{-1} rotenone'+', 0.15 mmol l^{-1} NADH, 0.3 mmol l^{-1} KCN, 0.06 mmol l^{-1} coenzyme Q10 and 3 mg ml^{-1} BSA; (2) complex II [CII; ε=21.9 (mmol l^{-1})$^{-1}$ cm^{-1} at 600 nm]: 20 mmol l^{-1} succinate'−', 0.3 mmol l^{-1} KCN, 0.05 mmol l^{-1} dichlorophenolindophenol and 0.05 mmol l^{-1} decylubiquinone (Enzo Life Sciences, Farmingdale, NY, USA); (3) complex III [CIII; ε=28.5 (mmol l^{-1})$^{-1}$ cm^{-1} at 550 nm]: 0.075 mmol l^{-1} oxidized cytochrome *c* (Calzyme Laboratories, San Luis Obispo, CA, USA), 0.05 mmol l^{-1} KCN,

0.1 mmol l^{-1} EDTA, 0.1 mmol l^{-1} decylubiquinol (Enzo Life Sciences); (4) complex IV [CIV; ε=28.5 (mmol l^{-1})$^{-1}$ cm^{-1} at 550 nm]: 0.2 mmol l^{-1} reduced cytochrome *c*'−' (Calzyme Laboratories); and (5) F_oF_1-ATP synthase [ATPsyn; ε=6.22 (mmol l^{-1})$^{-1}$ cm^{-1} at 340 nm]: 0.005 mmol l^{-1} oligomycin'+' (Enzo Life Sciences), 3 mmol l^{-1} Mg·ADP, 10 mmol l^{-1} $MgCl_2$, 10 mmol l^{-1} glucose, 1.5 mmol l^{-1} $NADP^+$, 1 U hexokinase (Roche, Basel, Switzerland), 1 U glucose-6-phosphate dehydrogenase.

For carbohydrate metabolism: (6) hexokinase [HK; ε=6.22 (mmol l^{-1})$^{-1}$ cm^{-1} at 340 nm]: 10 mmol l^{-1} glucose'−', 3 mmol l^{-1} Mg·ATP, 10 mmol l^{-1} $MgCl_2$, 1.5 mmol l^{-1} $NADP^+$, 1 unit of glucose-6-phosphate dehydrogenase; (7) phosphofructokinase [PFK; ε=6.22 (mmol l^{-1})$^{-1}$ cm^{-1} at 340 nm]: 10 mmol l^{-1} fructose-6-phosphate'−', 2.5 mmol l^{-1} Mg·ATP, 10 mmol l^{-1} $MgCl_2$, 0.15 mmol l^{-1} NADH, 6 mmol l^{-1} Mg·AMP, 0.3 mmol l^{-1} KCN, 1 unit of aldolase, 1 unit of triosephosphate isomerase, 1 unit of glyceraldehyde 3-phosphate dehydrogenase (Roche); (8) pyruvate kinase [PK; ε=6.22 (mmol l^{-1})$^{-1}$ cm^{-1} at 340 nm): 10 mmol l^{-1} phosphoenolpyruvate'−', 2.5 mmol l^{-1} Mg·ADP, 10 mmol l^{-1} $MgCl_2$, 0.15 mmol l^{-1} NADH, 1 unit of lactate dehydrogenase (Roche); (9) lactate dehydrogenase [LDH; ε=6.22 (mmol l^{-1})$^{-1}$ cm^{-1} at 340 nm]: 5 mmol l^{-1} pyruvate'−', 0.15 mmol l^{-1} NADH.

For the tricarboxylic acid cycle: (10) citrate synthase [CS; ε=14.15 (mmol l^{-1})$^{-1}$ cm^{-1} at 412 nm]: 0.5 mmol l^{-1} oxaloacetate'−', 0.15 mmol l^{-1} acetyl-coA, 0.15 mmol l^{-1} 5,5′-dithiobis-2-nitrobenzoic acid; (11) isocitrate dehydrogenase [IDH; ε=6.22 (mmol l^{-1})$^{-1}$ cm^{-1} at 340 nm]: 5 mmol l^{-1} isocitrate'−', 1.5 mmol l^{-1} $NADP^+$; (12) malate dehydrogenase [MDH; ε=6.22 (mol l^{-1})$^{-1}$ cm^{-1} at 340 nm]: 0.5 mmol l^{-1} oxaloacetate'−', 0.15 mmol l^{-1} NADH.

For fatty acid metabolism: (13) 3-hydroxyacyl-CoA dehydrogenase [HOAD; ε=6.22 (mmol l^{-1})$^{-1}$ cm^{-1} at 340 nm]: 0.15 mmol l^{-1} acetoacetyl CoA'−', 0.15 mmol l^{-1} NADH; (14) carnitine palmitoyltransferase [CPT; ε=14.15 (mmol l^{-1})$^{-1}$ cm^{-1} at 412 nm]: 5 mmol l^{-1} L-carnitine'−', 0.2 mmol l^{-1} 5,5′-dithiobis-2-nitrobenzoic acid, 0.1 mmol l^{-1} palmitoyl CoA.

For adenylate metabolism: (15) adenylate kinase [AK; ε=6.22 (mmol l^{-1})$^{-1}$ cm^{-1} at 340 nm]: 3 mmol l^{-1} Mg·ADP'−', 10 mmol l^{-1} $MgCl_2$, 10 mmol l^{-1} glucose, 1.5 mmol l^{-1} $NADP^+$, 1 unit of hexokinase (Roche), 1 unit of glucose-6-phosphate dehydrogenase; (16) creatine kinase [CK; ε=6.22 (mmol l^{-1})$^{-1}$ cm^{-1} at 340 nm]: 10 mmol l^{-1} creatine phosphate'−', 5 mmol l^{-1} Mg·ATP, 10 mmol l^{-1} glucose, 1.5 mmol l^{-1} $NADP^+$, 1 unit of hexokinase (Roche), 1 unit of glucose-6-phosphate dehydrogenase.

Enzyme activities are expressed in units of micromole substrate per milligram tissue protein per minute, with protein concentrations determined using the BCA method (Sigma-Aldrich). Preliminary experiments determined that all substrate concentrations were saturating. Assays were measured using a SpectraMax Plus 384 spectrophotometer (Molecular Devices, Sunnyvale, CA, USA). Data were analyzed using the accompanying SoftMax Pro 6.3 program and GraphPad Prism 6 (GraphPad Software, La Jolla, CA, USA). Biochemicals were from Sigma-Aldrich unless otherwise stated.

Myoglobin content assays on homogenized muscles

The Mb content of muscle homogenates was determined using a modified version of the original method (Reynafarje, 1962). Samples of frozen pectoralis, gastrocnemius and left ventricle muscle were homogenized in 19.25 volumes of ice-cold homogenizing buffer B (40 mmol l^{-1} KH_2PO_4 buffer, pH 6.6). Homogenates were then centrifuged at 13,700 ***g*** at 4°C for 99 min and the supernatant collected. Preliminary experiments confirmed that the values obtained by spinning at 13,700 ***g*** for 99 min were equivalent to those obtained by spinning at 28,000 ***g*** for 50 min, the conditions originally proposed by Reynafarje (1962). The supernatant was carefully transferred to a glass tonometer and exposed to pure carbon monoxide (CO) for 8 min under continuous rotation. Sodium dithionite was then added to ensure complete reduction of the sample, and subjected to a further 2 min of CO exposure under continuous rotation. The samples were then diluted 19.5-fold and carefully transferred to a 1 ml cuvette. The cuvette was measured in a SpectraMax Plus 384 spectrophotomer (Molecular Devices) at 538 and 568 nm using homogenization buffer B as a blank. Mb content (mg g^{-1} tissue) was determined using the following calculation (Reynafarje, 1962):

$$[\mathrm{Mb}] = (\mathrm{OD}_{538} - \mathrm{OD}_{568}) \times 112.6.$$

Mb content was assayed in triplicate. Data were analyzed using the accompanying SoftMax Pro 6.3 program and GraphPad Prism 6 (GraphPad Software).

Statistical analysis

The main effects of respiration state and population altitude on mitochondrial respiration were evaluated using two-factor ANOVA followed by the Bonferroni *post hoc* tests to compare between populations within each respiration state (means±s.e.m.). Enzyme activities and Mb content were compared between highland and lowland ducks using a two-tailed Student's *t*-test (means±s.e.m.). A probability of $P<0.05$ was considered significant.

RESULTS

Respiration of permeabilized muscle fibers

The respiratory capacities for oxidative phosphorylation (oxphos) in the gastrocnemius muscle was significantly higher in the high-altitude population (Fig. 1A), as reflected by a significant main effect of altitude in two-factor ANOVA ($P=0.015$, $N=6$). Respiration rates with pyruvate and malate (via oxphos CI) were 1.8-fold higher in the high-altitude population ($P<0.05$, $N=6$), but subsequent addition of glutamate did not have a significant effect on respiration rate. Addition of succinate to stimulate oxphos CI+CII increased respiration, with the rate being 1.7-fold higher in the high-altitude population ($P<0.05$, $N=6$). Maximal respiration via oxphos CIV, achieved with the addition of TMPD and ascorbate, was 2.1-fold higher in the high-altitude population ($P<0.05$, $N=6$). Leak-state respiration was significantly higher at 0.70±0.045 nmol mg^{-1} min^{-1} for the high-altitude population and 0.37±0.034 nmol mg^{-1} min^{-1} for the low-altitude population ($P=0.002$, $N=6$). The factorial increase in respiration in the transition from leak state to oxphos state represents the acceptor control ratio (ACR), which was not significantly different between populations (high-altitude ACR=4.66±0.85, low-altitude ACR=4.68±0.46).

The respiratory capacities for oxphos did not differ between altitudes in the pectoralis (Fig. 1B: $P=0.95$, $N=6$) or in the left ventricle (Fig. 1C: $P=0.43$, $N=6$). Respiration rates with pyruvate and malate (via oxphos CI) were not significantly different between populations in either tissue. Similar to what was observed in the gastrocnemius, the subsequent addition of glutamate had no significant effect on respiration rate. Addition of succinate to stimulate oxphos CI+CII increased respiration in both tissues, but

Journal of Experimental Biology (2016) 219, 3719-3728 doi:10.1242/jeb.142711

there were no significant differences between populations. Maximal respiration via oxphos CIV, achieved with the addition of TMPD and ascorbate, was also not significantly different between populations in either tissue. Leak-state respiration (pectoralis, 0.76±0.145 nmol mg^{-1} min^{-1} for the high-altitude population and 0.59±0.09 nmol mg^{-1} min^{-1} for the low-altitude population, P=0.35; left ventricle, 1.42±0.62 nmol mg^{-1} min^{-1} for the high-altitude population and 0.76±0.27 nmol mg^{-1} min^{-1} for the low-altitude population, P=0.10) and ACR (pectoralis, high-altitude ACR=5.27±1.18, low-altitude ACR=5.59±0.61; left ventricle, high-altitude ACR=3.84±0.25, low-altitude ACR=4.01±0.19) were similar between populations in both tissues.

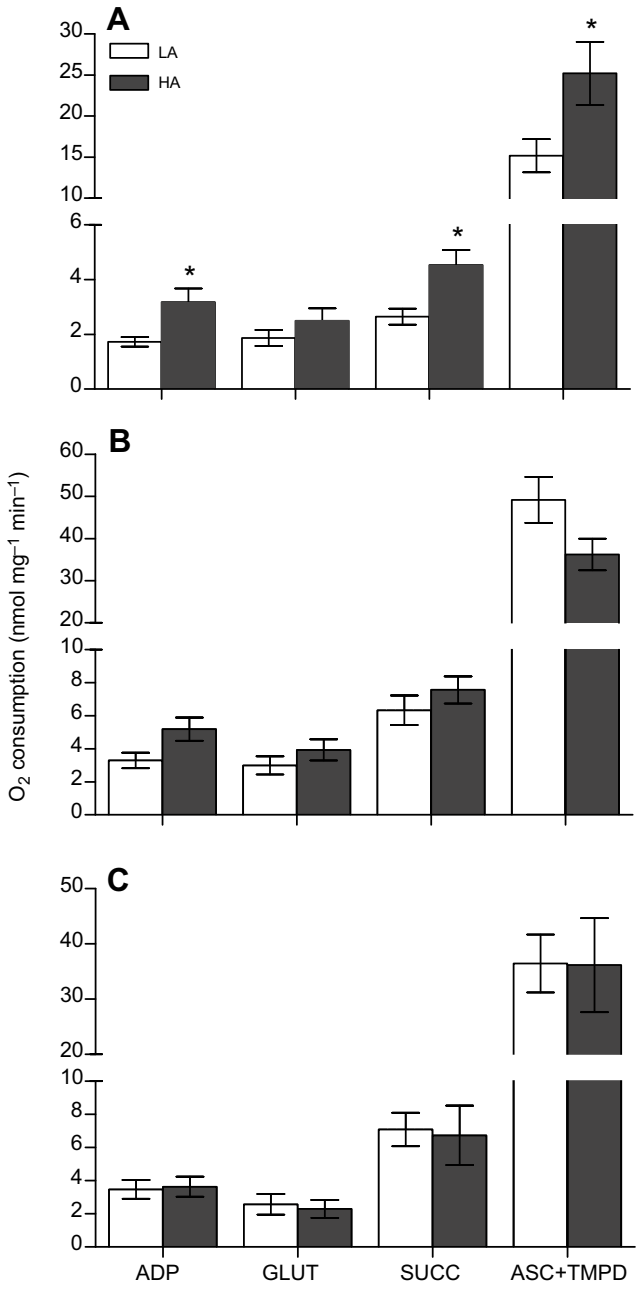

Fig. 1. Respiration of permeabilized fibers, measured in the gastrocnemius, left ventricle and pectoralis of the torrent duck (*Merganetta armata*). (A) Gastrocnemius; (B) left ventricle; (C) pectoralis. Respiration rates were measured in the presence of pyruvate, malate and the following: ADP; ADP and glutamate (GLUT); ADP, glutamate and succinate (SUCC); ADP, glutamate, succinate, ascorbate and TMPD (ASC+TMPD). Bars represent the respiration rates of the low-altitude (LA; white) and high-altitude (HA; black) populations. Values are given as the means±s.e.m. (N=6). *Significantly different from the corresponding low-altitude value (Student's t-test; P<0.05).

Differences in high-altitude muscle enzyme activities

In concert with the differences in mitochondrial respiratory capacities, the maximal activities of several enzymes involved in determining oxidative and glycolytic capacities were significantly greater in the gastrocnemius of the high-altitude population compared to the lowland population (Fig. 2). COX, the terminal oxygen acceptor of the electron transport chain, exhibited the largest difference (2.5-fold) in activity between populations. The citric acid cycle enzyme MDH was also more active in the highland population (by 31%), and the glycolytic enzymes PFK and PK were 22% and 30% greater in highlanders (P<0.05).

Although there were no differences in mitochondrial capacities in the left ventricle, enzymatic analysis demonstrated significant differences in the maximal activities of at least one enzyme in every facet of metabolism studied. Seven enzymes were observed to have different maximal activities in the left ventricle between populations (Fig. 3). The activity of five enzymes had higher maximal activities in the high-altitude population: LDH, an enzyme involved in carbohydrate metabolism, was 31% greater; the citric acid cycle enzyme MDH was 35% greater; ATP synthase was 43% greater; HOAD, an enzyme involved in fatty acid metabolism, was 49% greater; and CK, an enzyme involved in adenylate metabolism, was 128% greater (P<0.05). Conversely, two enzymes had lower maximal activities in the high-altitude population: the electron transport chain enzyme CIII was 28% lower, whereas AK, an enzyme involved in adenylate metabolism, was 23% lower (P<0.05).

Consistent with the lack of variation in mitochondrial respiratory capacities in the pectoralis, the maximal activity of only one enzyme differed significantly in the pectoralis between the high- and low-altitude populations. The activity of MDH, a major enzyme of the citric acid cycle, was 26% higher in the pectoralis in the high-altitude torrent ducks (P<0.05). Fig. 4 shows that, interestingly, the

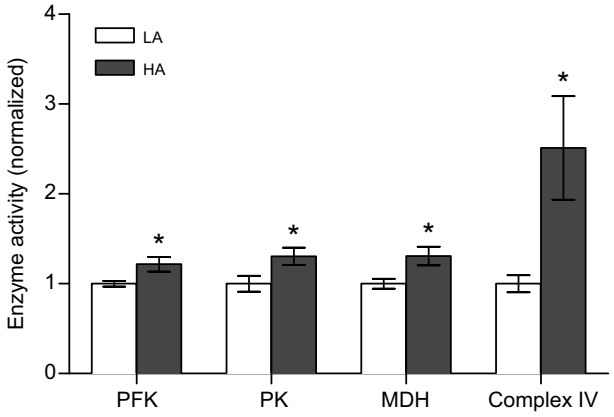

Fig. 2. Differences in enzyme activity from samples of the gastrocnemius of the torrent duck (*Merganetta armata*). White bars represent the low-altitude (LA) population; black bars represent the high-altitude (HA) population. Values are given as the means±s.e.m. (N=6). *Significantly different from the corresponding low-altitude value (Student's t-test; P<0.05). PFK, phosphofructokinase; PK, pyruvate kinase; MDH, malate dehydrogenase.

Journal of Experimental Biology

Journal of Experimental Biology (2016) 219, 3719-3728 doi:10.1242/jeb.142711

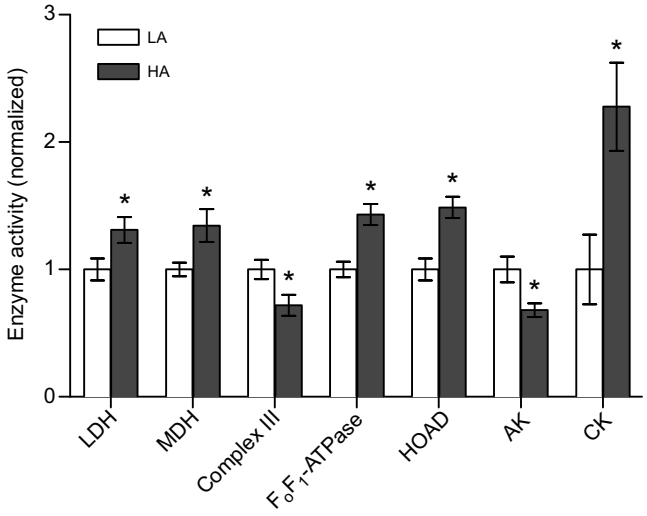

Fig. 3. Differences in enzyme activity from samples of the left ventricle of the torrent duck (*Merganetta armata*). White bars represent the low-altitude (LA) population; black bars represent the high-altitude (HA) population. Values are given as the means±s.e.m. (N=6). *Significantly different from the corresponding low-altitude value (Student's t-test; P<0.05). LDH, lactate dehydrogenase; MDH, malate dehydrogenase; HOAD, 3-hydroxyacyl-CoA dehydrogenase; AK, adenylate kinase; CK, creatine kinase.

maximal activity of MDH was similarly enhanced in all muscles tested (+35% in the left ventricle; +31% in the gastrocnemius).

Many enzymes had similar activities between high- and low-altitude populations in left ventricle, pectoralis and gastrocnemius muscle (Table 1), and the measured activities were generally comparable to previous measurements in waterfowl and other bird species (Farrar and Farrar, 1983; Farrar et al., 1983; Turner and Butler, 1988; Bishop et al., 1995). Soluble-protein content in tissue extracts did not differ significantly between populations in any tissue. Mean protein concentrations in the high-altitude population were 10.61±0.50, 7.06±0.33 and 7.88±0.23 mg g^{-1} wet mass and in the low-altitude population were 10.71±0.51, 6.58±0.38 and 7.45±0.42 mg g^{-1} wet mass (means±s.e.m., N=6) in the pectoralis, left ventricle and gastrocnemius, respectively.

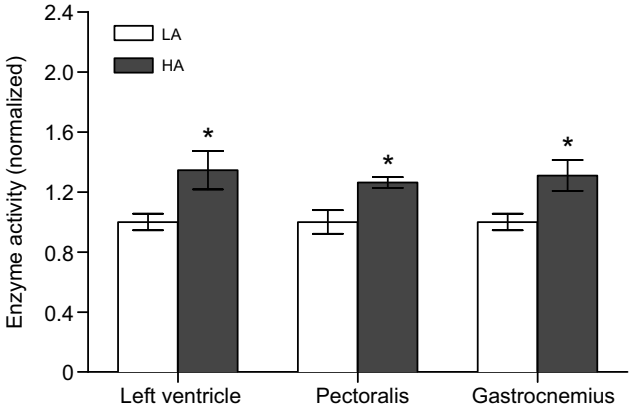

Fig. 4. Differences in malate dehydrogenase (MDH) enzyme activity from samples of left ventricle, pectoralis and gastrocnemius of the torrent duck (*Merganetta armata*). White bars represent the low-altitude (LA) population; black bars represent the high-altitude (HA) population. Values are given as the means±s.e.m. (N=6). *Significantly different respiration in high-altitude torrent ducks in comparison to low-altitude torrent ducks (two-way ANOVA with Bonferroni *post hoc* test; P<0.05).

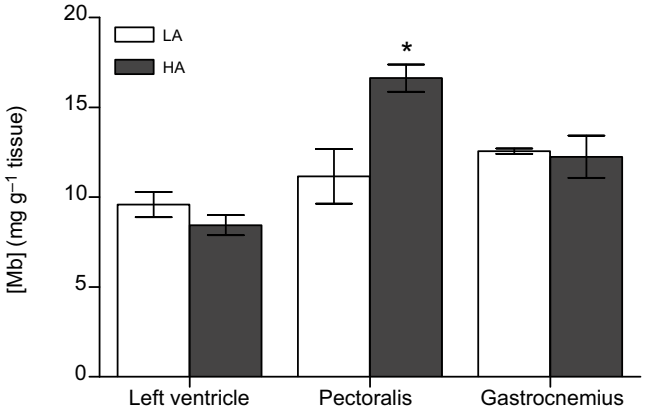

Fig. 5. Myoglobin (Mb) concentrations of the left ventricle, pectoralis and gastrocnemius of the torrent duck (*Merganetta armata*). Bars represent the protein content in tissue of the low-altitude (LA; white) and high-altitude (HA; black) populations. Values are given as the means±s.e.m. (N=6). *Significantly different respiration in high-altitude torrent ducks in comparison to low-altitude torrent ducks (Student's t-test; P<0.05).

Myoglobin content assays on homogenized muscles

Mb content was ~30% greater in the pectoralis muscle of high-altitude ducks, but was similar between populations in the other muscle tissues (Fig. 5). Mb content was the highest in the gastrocnemius of the low-altitude population compared with other muscles, whereas the high-altitude population had the highest concentration in the pectoralis. Surprisingly, the left ventricle had lower concentrations of Mb than the other two muscle tissues in both populations.

DISCUSSION

Life at high altitude imposes many challenges on the animals that inhabit these environments. One of the toughest challenges to overcome for life at high altitudes is matching O_2 supply to demand in the face of a lower environmental P_{O_2}. Our present findings suggest that high-altitude torrent ducks have an enhanced respiratory capacity in the gastrocnemius muscle – the major locomotor muscle for underwater swimming – associated with heightened glycolytic capacity and CIV activity, which likely facilitates mitochondrial respiration under hypoxic conditions. Compared with low-altitude torrent ducks, they also exhibit differences in enzyme activities in heart and pectoralis muscles that are seemingly unrelated to variation in respiratory capacity, but may be otherwise important for sustaining muscle function and exercise performance at high altitude. Overall, our work suggests that life at high altitudes is associated with physiological and biochemical alterations to several muscles in the torrent duck, which may be essential for this species' exceptional ability to thrive in the fast-moving high-altitude streams of the Peruvian Andes.

Enhanced aerobic capacity in the gastrocnemius muscle of highland torrent ducks

The enhanced respiratory capacity in the gastrocnemius muscle of highland torrent ducks (Fig. 1A) could be important at high altitudes for increasing the thermogenic capacity for heat production in the cold, and/or increasing hypoxia tolerance. Birds meet the bulk of the demand for thermogenesis by shivering (West, 1965; Bicudo, 1996). Non-shivering thermogenesis may play a role in thermoregulation in ducklings (Teulier et al., 2010), but the relative importance of non-shivering thermogenesis in most species of birds is unclear (Barré et al., 1989; Connolly et al.,

Journal of Experimental Biology (2016) 219, 3719-3728 doi:10.1242/jeb.142711

Table 1. Maximal activities (μmol mg^{-1} protein min^{-1}) of metabolic enzymes from the left ventricle, pectoralis and gastrocnemius of *Merganetta armata*

	Left ventricle		Pectoralis		Gastrocnemius	
Enzyme	LA	HA	LA	HA	LA	HA
Carbohydrate metabolism						
HK	0.134±0.014	0.155±0.011	0.033±0.003	0.033±0.004	0.073±0.006	0.085±0.010
PFK	2.883±0.143	2.815±0.178	2.307±0.115	2.571±0.173	3.217±0.105	3.915±0.265*
PK	18.60±1.929	18.80±1.028	50.00±2.404	47.78±3.279	37.54±3.358	48.97±3.656*
LDH	23.40±2.015	30.66±2.350*	48.25±3.012	48.56±3.743	41.78±3.942	49.24±4.458
Citric acid cycle						
CS	11.70±0.672	12.09±0.398	9.465±1.046	9.641±0.652	5.413±0.447	6.107±0.420
IDH	2.998±0.174	3.202±0.192	2.821±0.161	2.998±0.174	2.326±0.150	2.498±0.145
MDH	194.2±10.53	261.2±24.98*	155.8±12.23	196.6±5.595*	148.6±8.159	194.7±15.35*
Electron transport chain						
CI	0.211±0.033	0.205±0.021	0.125±0.024	0.110±0.013	0.164±0.007	0.189±0.016
CII	0.827±0.050	0.776±0.025	0.429±0.049	0.452±0.044	0.474±0.046	0.495±0.061
CIII	0.375±0.028	0.269±0.031*	0.327±0.015	0.372±0.045	0.399±0.048	0.431±0.090
CIV	0.592±0.062	0.591±0.060	0.556±0.047	0.534±0.112	0.736±0.071	1.847±0.424*
ATPsyn	14.05±0.857	20.12±1.147*	14.52±2.422	17.11±2.943	9.909±1.373	10.91±2.290
Fatty acid metabolism						
HOAD	0.370±0.032	0.550±0.031*	0.215±0.040	0.209±0.019	0.355±0.022	0.362±0.022
CPT	0.057±0.005	0.064±0.007	0.055±0.002	0.060±0.004	0.053±0.006	0.063±0.011
Adenylate metabolism						
AK	7.380±0.742	5.019±0.395*	9.818±1.121	8.642±0.868	16.15±1.603	15.14±2.856
CK	1.622±0.443	3.695±0.562*	2.178±0.202	2.396±0.240	4.615±0.592	4.164±0.257

LA, low altitude; HA, high altitude; HK, hexokinase; PFK, phosphofructokinase; PK, pyruvate kinase; LDH, lactate dehydrogenase; CS, citrate synthase; IDH, isocitrate dehydrogenase; MDH, malate dehydrogenase; CI, complex I; CII, complex II; CIII, complex III; CIV, complex IV; ATPsyn, F_oF_1-ATP synthase; HOAD, 3-hydroxyacyl-CoA dehydrogenase; CPT, carnitine palmitoyltransferase; AK, adenylate kinase; CK, creatine kinase.
Values are given as the means±s.e.m. (*N*=6).
*Significantly different activity in high-altitude torrent ducks in comparison to low-altitude torrent ducks (Student's *t*-test; $P<0.05$).

1989). Regardless of whether shivering or non-shivering thermogenesis predominates, the flight muscles (particularly the pectoralis and the supracoracoideus) are believed to be a major site of thermogenesis in adult birds (Petit and Vézina, 2014; Block, 1994; Bicudo et al., 2002). The thigh muscles are very important for thermogenesis in early development, but their relative importance decreases as the flight muscles grow and eventually reach a much larger mass (Marjoniemi and Hohtola, 2000; Sirsat et al., 2016). Therefore, although the increase in aerobic capacity in the gastrocnemius could be important for facilitating thermogenesis, it is curious that similar increases do not also occur in the pectoralis. Increases in oxidative capacity might have instead arisen to promote hypoxia resistance at high altitudes, a theory that has been suggested in other high-altitude taxa (Hochachka, 1985; Scott et al., 2009a,b; Lui et al., 2015). This theory suggests that, when the maximum attainable respiration of an individual muscle fiber is impaired from declines in intracellular O_2 tension, a higher oxidative capacity should increase the total mitochondrial O_2 flux of the entire muscle and thus help offset the inhibitory effects of hypoxia. This mechanism might be acting in torrent ducks to overcome intracellular hypoxia in the gastrocnemius muscle during swimming or diving at high altitude.

The enhanced respiratory capacity of highland torrent ducks was associated with greater activity of CIV, without any significant differences from lowland ducks in the activities of other electron transport chain enzymes (Table 1; Fig. 2). This was somewhat surprising in light of the common perception that CIV is generally in excess capacity, and that CIV exerts less control over pathway flux than other mitochondrial complexes (Telford et al., 2009). Although this could imply that relatively large increases in CIV activity are needed to achieve relatively small changes in mitochondrial oxygen consumption, our results suggest that this is not the case; the relative differences in the highland population for mitochondrial respiration (1.7- to 2.1-fold) were nearly as large as those for CIV activity (2.5-fold). It is also possible that the larger relative excess of CIV activity in highland ducks helps increase the O_2 affinity of mitochondria, by reducing the catalytic turnover rate of each CIV enzyme (Gnaiger et al., 1998; Kudin et al., 2002), and thus helps sustain ATP synthesis in hypoxia. However, although unique specializations in the activity, structure and function of CIV have been observed in the locomotor muscles of several high-altitude taxa (Sheafor, 2003; Scott et al., 2011; Lui et al., 2015), it is not clear whether these specializations affect mitochondrial O_2 affinity (Scott et al., 2009a,b).

Major regulatory enzymes of glycolysis, PK and PFK, also had higher activity in the gastrocnemius of the high-altitude population compared with those from low altitudes (Table 1; Fig. 2). PK and PFK have been suggested to exert significant metabolic control over glycolytic pathway flux when assessed using metabolic control analysis (Vogt et al., 2002a,b), and they both catalyze irreversible reactions in the glycolytic pathway and have long been discussed as sites of allosteric regulation (Scrutton and Utter, 1968). This is in line with the enhanced glycolytic enzyme activities in the locomotory muscle of many high-altitude mammals, and likely serves to increase the capacity for producing ATP from carbohydrate oxidation (Semenza et al., 1994; Firth et al., 1994; McClelland et al., 1998; Schippers et al., 2012). This could be especially beneficial in high-altitude hypoxia because of the inherent O_2 savings associated with oxidizing carbohydrates instead of other metabolic fuels (McClelland et al., 1998). This advantage seems to have been favored by natural selection in highland mice from the Andes, which have a greater preference for carbohydrate oxidation than lowland mice during exercise, even when compared at similar altitudes and exercise intensities (Schippers et al., 2012). The increased glycolytic activities in torrent ducks may also be reflective of an increased capacity for using anaerobic metabolism during short diving bouts, which may supply lactate for oxidation in the heart (see below). Many diving animals show increased glycolytic capacity in the muscles to

Journal of Experimental Biology

Journal of Experimental Biology (2016) 219, 3719-3728 doi:10.1242/jeb.142711

support underwater locomotion (George and Ronald, 1973; Simon et al., 1974; Castellini et al., 1981), and it is predictable that the demands for anaerobic metabolism could be higher while diving in high-altitude hypoxia.

Metabolic enzyme activities in the heart and flight muscle of highland torrent ducks

The numerous differences in enzyme activities between torrent duck populations suggest that life at high altitudes leads to metabolic restructuring of the left ventricle. The high-altitude population had a high activity of LDH in the heart (Table 1; Fig. 3), similar to what has been observed in various tissues of species that experience cold and hypoxic/anoxic environments (Rosser and Hochachka, 1993; Dawson et al., 2013; Shahriari et al., 2013; Katzenback et al., 2014). However, LDH in the heart likely facilitates lactate oxidation rather than production, a process that our data suggests might be enhanced in highland torrent ducks (Brooks, 1998; Gladden, 2004; Brooks, 2009). There is increasing evidence for a role of mitochondrial LDH that helps support lactate oxidation in an intracellular lactate shuttle, likely in an effort to balance glycolytic production of lactate with mitochondrial oxidation (Jouaville et al., 1999; Brooks et al., 1999; Van Hall, 2000; Passarella et al., 2008; Lottes et al., 2015), and it is possible that the capacity of this shuttle is enhanced in highland torrent ducks. An increased capacity for lactate oxidation in the left ventricle may also help minimize the inhibitory effects of lactate on fatty acid oxidation (Bielefeld et al., 1985; Wolfe, 1998; Liu et al., 2009). This could act in concert with the greater HOAD activity of highland torrent ducks (Fig. 3), an observation that is consistent with findings in several other high-altitude taxa (Bigard et al., 1991; Léon-Velarde, 1993; Sheafor, 2003; Scott et al., 2009a,b), in order to increase the sustainable yield of ATP from β-oxidation.

The heightened activity of CK in the heart of highland torrent ducks might be important for facilitating energy supply. The greater CK activity in highland versus lowland torrent ducks is consistent with previous observations in Tibetan chickens, in which developmental hypoxia increased expression of mitochondrial CK in the heart (Li and Zhao, 2009). This response to hypoxia *in ovo* seemed to be unique to this highland strain of chicken, because lowland-strain chickens did not increase mitochondrial CK expression in the heart in response to the same developmental hypoxia exposure (Li and Zhao, 2009). Expression of mitochondrial CK is also elevated in the locomotory muscle of high-altitude deer mice compared to their low-altitude counterparts (Scott et al., 2015a,b). Increases in mitochondrial CK could augment the effectiveness of the CK shuttle – a mechanism that enhances the supply of ATP equivalents from mitochondria to myofibrils in cardiac and oxidative muscles (Ventura-Clapier et al., 1998). Measurements of creatine sensitivity of mitochondrial respiration have suggested that this shuttle is enhanced in the flight muscle of the high-altitude bar-headed goose (Scott et al., 2009a,b), and it is possible that the left ventricle of the highland torrent duck possesses similar mechanisms to improve the effectiveness of ATP supply and energy coupling.

Interestingly, only one enzyme, MDH, was similarly greater (26–35%) in the highland population across all three muscle types (Fig. 4). If this is caused by higher activities of both mitochondrial and cytosolic forms of MDH, it might suggest that the highland population has an increased reliance on the malate–aspartate shuttle for NADH transport across the mitochondrial membrane (Barron et al., 1998; Kane, 2014). It could also help regenerate NADH/NAD^+ redox ratio in the cytosol (Rubi et al., 2004), which might be beneficial during anaerobic dives as a means of maintaining cytosolic redox balance without relying on lactate production (which can come at the cost of a metabolic acidosis, which can hinder muscle function) or even the consumption of lactate either produced locally within the cell or from other tissues. The pectoralis, in particular, could act as a lactate sink given the possible high oxygen content due to generally high Mb content in the torrent duck. Although differences in MDH activity in the skeletal muscles could be partially explained by differences in fiber-type composition (Schantz and Henriksson, 1987; Essén-Gustavsson and Henriksson, 1984), this mechanism cannot explain the observed differences in the cardiac muscle. Furthermore, the lack of any variation in respiratory capacity in the pectoralis suggests that this particular muscle may have a similar fiber-type composition between highland and lowland populations of torrent duck.

High Mb content in torrent ducks

Mb content was generally quite high in torrent ducks, higher than many species of non-diving waterfowl but comparable to previous measurements in other species of diving birds (Pages and Planas, 1983). High Mb content is known to increase oxygen-storage capacity and may also promote intracellular oxygen transport (Wittenberg and Wittenberg, 2006; Koch and Britton, 2008; Mirceta et al., 2013). Diving animals rely heavily on Mb O_2 stores, rather than capillaries, for mitochondrial O_2 supply during dives, and dive duration is positively correlated with Mb concentration (Reed et al., 1994; Butler and Jones, 1997; Kooyman and Ponganis, 1998; Dolar et al., 1999; Helbo and Fago, 2012; Mirceta et al., 2013; Wright and Davis, 2015). Torrent ducks have nearly four times the Mb content reported for non-aquatic birds (Pages and Planas, 1983; Butler and Jones, 1997; Kooyman and Ponganis, 1998; Wright and Davis, 2015), suggesting that they may also have a strong dive capacity.

The heightened Mb content of highland torrent ducks in the pectoralis muscle may be important for improving mitochondrial O_2 supply to overcome hypoxia and support flight and/or thermogenesis at high altitude. In line with this idea, Mb content is augmented in the locomotory muscle of many high-altitude animals (Vaughan and Pace, 1956; Reynafarje, 1963; Reynafarje and Morrison, 1962; Moore et al., 2002; Xin et al., 2015). Alternatively, it may provide an added store of O_2 to support the pectoralis muscle during dives, in order to minimize the blood-flow demands of this tissue (either during or between dive bouts) or to reduce the magnitude of metabolic acidosis.

Potential importance of evolved trait differences versus plasticity

Differences between highland and lowland torrent ducks could arise as adaptations to high altitude or from plasticity (developmental or adult) resulting from living in a high-altitude environment. However, there is very little genetic variation across altitudinal gradients in torrent ducks and gene flow between high and low altitudes is likely significant (McCracken et al., 2009a,b; Natarajan et al., 2015; K.G.M., unpublished). Therefore, if the observed differences in muscle physiology in highland torrent ducks arose as evolutionary adaptations to the high-altitude environment, then these differences are presumably under very strong selection. This is not beyond possibility, as significant differences in muscle phenotype are known to exist in other highland taxa when compared in common environments (Scott et al., 2009a,b; Lui et al., 2015). It is possible that hypoxia exposure associated with breath-holding diving led to exaptations (pre-adaptations) for life at high altitudes in lowland

torrent ducks, and reduced the selective advantage for adaptive changes in some traits in the high-altitude population. Consistent with this idea, both high- and low-altitude populations of torrent ducks have hemoglobin with an affinity (P_{50}=33–35 mmHg) that is much greater than most birds (in which P_{50} typically ranges between 44 and 52 mmHg) and is comparable to that of the emperor penguin (P_{50}=28–36 mmHg) (Christensen and Dill, 1935; Hirsowitz et al., 1977; Lutz, 1980; Wastl and Leiner, 1931; Meir and Ponganis, 2009; Tamburrini et al., 1994; Natarajan et al., 2015). However, unlike highland populations of many other South American waterfowl, highland torrent ducks lack further evolved specialization in hemoglobin sequence or O_2-binding affinity compared to their lowland counterparts (McCracken et al., 2009a,b). The enhanced aerobic capacity of the torrent ducks could alternatively be a consequence of physiological plasticity in response to differences in behavior between populations or to the colder and/or hypoxic environment at high altitude. Chronic cold exposure in particular has been shown to increase muscle oxidative capacity in some studies to improve thermogenic capacity (Mineo et al., 2012). Whatever the ultimate cause, it is clear that significant changes in muscle physiology are important for life at high altitudes in the torrent duck.

Acknowledgements
Permits to conduct this research in Peru were provided by SERFOR (Permit: RDG no. 190-2015-SERFOR-DGGSPFFS), and authorization to work in the field was provided by the community of Villaycocha, and private landowners in Santa Rosa de Quives (Fundo Huanchuy and Hostiliano family).

Competing interests
The authors declare no competing or financial interests.

Author contributions
Conceptualization: N.J.D., K.G.M., G.R.S.; Methodology: N.J.D., K.G.M., G.R.S.; Formal analysis: N.J.D.; Investigation: N.J.D.; Resources: N.J.D., C.M.I., L.A., R.C., J.M.Y., B.C., W.K.M., K.G.M., G.R.S.; Writing - original draft preparation: N.J.D.; Writing - review and editing: N.J.D., C.M.I., L.A., R.C., J.M.Y., B.C., W.K.M., K.G.M., G.R.S.; Visualization: N.J.D.; Supervision: W.K.M., K.G.M., G.R.S.; Project administration: N.J.D., C.M.I., L.A., R.C., J.M.Y., B.C., W.K.M., K.G.M., G.R.S.; Funding acquisition: K.G.M., G.R.S.

Funding
The equipment and operational costs of this research was supported by funds from McMaster University, the Canada Foundation for Innovation, the Ontario Ministry of Research and Innovation, and Natural Sciences and Engineering Research Council of Canada (NSERC) Discovery Grants to G.R.S. and W.K.M. This research was also supported by a National Science Foundation (NSF) grant (IOS-0949439) to K.G.M. G.R.S. is supported by the Canada Research Chairs Program. N.J.D. is supported by the James A. Kushlan endowment (awarded to K.G.M.) from the University of Miami.

References
Baldassarre, G. A. and Bolen, E. G. (1994). *Waterfowl Ecology and Management* (ed. G. A. Baldassarre and E. G. Bolen), pp. 60-80. New York: John Wiley & Sons, Inc.

Barré, H., Duchamp, C., Rouanet, J.-L., Dittmar, A. and Delhomme, G. (1989). Muscular nonshivering thermogenesis in cold-acclimated ducklings. In *Physiology of Cold Adaptation in Birds* (ed. C. Bech and R. E. Reinertsen), pp. 49-58. New York: Plenum.

Barron, J. T., Gu, L. and Parrillo, J. E. (1998). Malate-aspartate shuttle, cytoplasmic NADH redox potential, and energetics in vascular smooth muscle. *J. Mol. Cell. Cardiol.* **30**, 1571-1579.

Bicudo, J. E. P. W. (1996). Physiological correlates of daily torpor in hummingbirds. In *Animals and Temperature: Phenotypic and Evolutionary Adaptation* (ed. I. A. Johnston and A. F. Bennet), pp. 293-311. Cambridge: Cambridge University Press.

Bicudo, J. E. P. W., Bianco, A. C. and Vianna, C. R. (2002). Adaptive thermogenesis in hummingbirds. *J. Exp. Biol.* **205**, 2267-2273.

Bielefeld, D. R., Vary, T. C. and Neely, J. R. (1985). Inhibition of carnitine palmitoyl-CoA transferase activity and fatty acid oxidation by lactate and oxfenicine in cardiac muscle. *J. Mol. Cell. Cardiol.* **17**, 619-625.

Bigard, A. X., Brunet, A., Guezennec, C. Y. and Monod, H. (1991). Skeletal muscle changes after endurance training at high altitude. *J. Appl. Physiol.* **71**, 2114-2121.

Bishop, C. M., Butler, P. J., Egginton, S., Elhaj, A. J. and Gabrielsen, G. W. (1995). Development of metabolic enzyme activity in locomotor and cardiac muscles of the migratory barnacle goose. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **269**, R64-R72.

Block, B. A. (1994). Thermogenesis in muscle. *Annu. Rev. Physiol.* **56**, 535-577.

Brooks, G. A. (1998). Mammalian fuel utilization during sustained exercise, *Comp. Biochem. Phys. B* **120**, 89-107.

Brooks, G. A. (2009). Cell-cell and intracellular lactate shuttles. *J. Physiol.* **587**, 5591-5600.

Brooks, G. A., Dubouchaud, H., Brown, M., Sicurello, J. P. and Butz, C. E. (1999). Role of mitochondrial lactate dehydrogenase and lactate oxidation in the intracellular lactate shuttle. *Proc. Natl. Acad. Sci. USA* **96**, 1129-1134.

Butler, P. J., & Jones, D. R. (1997). Physiology of diving of birds and mammals. *Physiological reviews*, **77**, 837-899.

Carboneras, C. (1992). Family *Anatidae* (ducks, geese and swans). In *Handbook of the Birds of the World* (ed. J. del Hoyo, A. Elliott and J. Sargatal), p. 599. Barcelona: Lynx Edicions.

Castellini, M. A., Somero, G. N. and Kooyman, G. L. (1981). Glycolytic enzyme activities in tissues of marine and terrestrial mammals. *Physiol. Zool.* **54**, 242-252.

Christensen, E. H. and Dill, D. B. (1935). Oxygen dissociation curves of bird blood. *J. Biol. Chem.* **109**, 443-448.

Connolly, E., Nedergaard, J. and Cannon, B. (1989). Shivering and nonshivering thermogenesis in birds: A mammalian view. In *Physiology of Cold Adaptation in Birds*, (pp. 37-48). Springer US.

Dawson, N. J., Bell, R. A. V. and Storey, K. B. (2013). Purification and properties of white muscle lactate dehydrogenase from the anoxia-tolerant turtle, the red eared slider, *Trachemys scripta elegans*. *Enz. Res.* **2013**, 784973.

Dolar, M. L., Suarez, P., Ponganis, P. J. and Kooyman, G. L. (1999). Myoglobin in pelagic small cetaceans. *J. Exp. Biol.* **202**, 227-236.

Essén-Gustavsson, B. and Henriksson, J. (1984). Enzyme levels in pools of microdissected human muscle fibres of identified type: adaptive response to exercise. *Acta Physiol. Scand.* **120**, 505-515.

Farrar, W. W. and Farrar, Y. J. (1983). Energy enzymes from the flight muscle of the house sparrow *Passer domesticus*—I. Activity levels of the glycolytic enzymes from the soluble fraction. *Comp. Biochem. Physiol. B Comp. Biochem.* **74**, 549-551.

Farrar, W. W., Farrar, Y. J. and Perry, R. (1983). Energy enzymes from the flight muscle of the house sparrow, *Passer domesticus*—II. Physical and chemical properties related to purification of the glycolytic enzymes. *Comp. Biochem. Physiol. B* **75**, 317-321.

Feinsinger, R., Colwell, R. K., Terborgh, J. and Chaplin, S. B. (1979). Elevation and the morphology, flight energetics, and foraging ecology of tropical hummingbirds. *Am. Nat.* **113**, 481-497.

Firth, J. D., Ebert, B. L., Pugh, C. W., & Ratcliffe, P. J. (1994). Oxygen-regulated control elements in the phosphoglycerate kinase 1 and lactate dehydrogenase A genes: similarities with the erythropoietin 3'enhancer. *Proceedings of the National Academy of Sciences*, **91**, 6496-6500.

George, J. C. and Ronald, K. (1973). The harp seal, *Pagophilus groenlandicus* (Erxleben, 1777). XXV. Ultrastructure and metabolic adaptation of skeletal muscle. *Can. J. Zool.* **51**, 833-839.

Gladden, L. B. (2004). Lactate metabolism: a new paradigm for the third millennium. *J. Physiol.* **558**, 5-30.

Gnaiger, E., Lassnig, B., Kuznetsov, A., Rieger, G. and Margreiter, R. (1998). Mitochondrial oxygen affinity, respiratory flux control and excess capacity of cytochrome c oxidase. *J. Exp. Biol.* **201**, 1129-1139.

Gutiérrez-Pinto, N., McCracken, K. G., Alza, L., Tubaro, P., Kopuchian, C., Astie, A. and Cadena, C. D. (2014). The validity of ecogeographical rules is context-dependent: testing for Bergmann's and Allen's rules by latitude and elevation in a widespread Andean duck. *Biol. J. Linn. Soc.* **111**, 850-862.

Hawkes, L.A., Balachandran, S., Batbayar, N., Butler, P.J., Chua, B., Douglas, D.C., Frappell, P.B., Hou, Y., Milsom, W.K., Newman, S.H. and Prosser, D.J. (2013). The paradox of extreme high-altitude migration in bar-headed geese Anser indicus. In *Proc. R. Soc. B* (Vol. 280, No. 1750, p. 20122114).

Helbo, S. and Fago, A. (2012). Functional properties of myoglobins from five whale species with different diving capacities. *J. Exp. Biol.* **215**, 3403-3410.

Hirsowitz, L. A., Fell, K. and Torrance, J. D. (1977). Oxygen affinity of avian blood. *Respir. Physiol.* **31**, 51-62.

Hochachka, P. W. (1985). Exercise limitations at high altitude: the metabolic problem and search for its solution. In *Circulation, Respiration, and Metabolism* (ed. G. R. Berlin), pp. 240-249. Germany: Springer-Verlag.

Jessen, T. H., Weber, R. E., Fermi, G., Tame, J. and Braunitzer, G. (1991). Adaptation of bird hemoglobins to high altitudes: demonstration of molecular mechanism by protein engineering. *Proc. Natl. Acad. Sci. USA* **88**, 6519-6522.

Johnsgard, P. A. (1968). Torrent duck. In *Waterfowl, THEIR Biology and Natural History* (ed. P. A. Johnsgard), pp. 60-65. Lincoln: University of Nebraska Press.

Johnsgard, P. A. (1972). Torrent ducks of the andes. In *Animals: The International Wildlife Magazine* (ed. P. A. Johnsgard), pp. 80-83. London: Nigel Sitwell Ltd.

Johnsgard, P. A. (1992). Torrent duck. In *Ducks in the Wild: Conserving Waterfowl and their Habitats* (ed. P. A. Johnsgard), pp. 70-72. Schrewsbury, England: Swan Hill Press.
Johnson, A. W. and Moffett, G. (1972). Torrent duck. In *Supplement to the Birds of Chile and Adjacent Regions of Argentina, Bolivia and Peru* (ed. A. W. Johnson), pp. 68-70. Beunos Aires: A.W. Johnson.
Jouaville, L. S., Pinton, P., Bastianutto, C., Rutter, G. A. and Rizzuto, R. (1999). Regulation of mitochondrial ATP synthesis by calcium: Evidence for a long-term metabolic priming. *Proc. Natl. Acad. Sci. USA* **96**, 13807-13812.
Kane, D. A. (2014). Lactate oxidation at the mitochondria: a lactate-malate-aspartate shuttle at work. *Front. Neurosci.* **8**, 366.
Katzenback, B. A., Dawson, N. J. and Storey, K. B. (2014). Purification and characterization of a urea sensitive lactate dehydrogenase from the liver of the African clawed frog, *Xenopus laevis*. *J. Comp. Physiol. B* **184**, 601-611.
Koch, L. G., & Britton, S. L. (2008). Aerobic metabolism underlies complexity and capacity. *J. Physiol.* **586**, 83-95.
Koepcke, M. (1970). Torrent duck. In *Birds of the Department of Lima, Peru* (ed M. Koepcke), pp. 37-39. Wynnewood, PA: Livingston Publishing Co.
Kooyman, G. L. and Ponganis, P. J. (1998). The physiological basis of diving to depth: birds and mammals. *Annu. Rev. Physiol.* **60**, 19-32.
Kudin, A., Vielhaber, S., Elger, C. E. and Kunz, W. S. (2002). Differences in flux control and reserve capacity of cytochrome c oxidase (COX) in human skeletal muscle and brain suggest different metabolic effects of mild COX deficiencies. *Mol. Biol. Rep.* **29**, 89-92.
Lee, S. Y., Scott, G. R. and Milsom, W. K. (2008). Have wing morphology or flight kinematics evolved for extreme high altitude migration in the bar-headed goose? *Comp. Biochem. Physiol. C Pharmacol. Toxicol.* **148**, 324-331.
León-Velarde, F., Sanchez, J., Bigard, A. X., Brunet, A., Lesty, C. and Monge, C. (1993). High altitude tissue adaptation in Andean coots: capillarity, fibre area, fibre type and enzymatic activities of skeletal muscle. *J. Comp. Physiol. B* **163**, 52-58.
Li, M. and Zhao, C. (2009). Study on Tibetan chicken embryonic adaptability to chronic hypoxia by revealing differential gene expression in heart tissue. *Sci. China C Life Sci.* **52**, 284-295.
Liu, C., Wu, J., Zhu, J., Kuei, C., Yu, J., Shelton, J., Sutton, S. W., Li, X., Yun, S. J., Mirzadegan, T. et al. (2009). Lactate inhibits lipolysis in fat cells through activation of an orphan G-protein-coupled receptor, GPR81. *J. Biol. Chem.* **284**, 2811-2822.
Lottes, R. G., Newton, D. A., Spyropoulos, D. D. and Baatz, J. E. (2015). Lactate as substrate for mitochondrial respiration in alveolar epithelial type II cells. *Am. J. Physiol. Lung Cell. Mol. Physiol.* **308**, L953-L961.
Lui, M. A., Mahalingam, S., Patel, P., Connaty, A. D., Ivy, C. M., Cheviron, Z. A., Storz, J. F., McClelland, G. B. and Scott, G. R. (2015). High-altitude ancestry and hypoxia acclimation have distinct effects on exercise capacity and muscle phenotype in deer mice. *Am. J. Physiol. Reg. Integr. Comp. Physiol.* **308**, R779-R791.
Lutz, P. L. (1980). On the oxygen affinity of bird blood. *Am. Zool.* **20**, 187-198.
Marjoniemi, K. and Hohtola, E. (2000). Does cold acclimation induce nonshivering thermogenesis in juvenile birds? Experiments with Pekin ducklings and *Japanese quail chicks*. *J. Com. Physiol. B* **170**, 537-543.
McClelland, G. B., Hochachka, P. W. and Weber, J.-M. (1998). Carbohydrate utilization during exercise after high-altitude acclimation: a new perspective. *Proc. Natl. Acad. Sci. USA* **95**, 10288-10293.
McCracken, K. G., Barger, C. P., Bulgarella, M., Johnson, K. P., Sonsthagen, S. A., Trucco, J., Valqui, T. H., Wilson, R. E., Winker, K. and Sorenson, M. D. (2009a). Parallel evolution in the major haemoglobin genes of eight species of Andean waterfowl. *Mol. Ecol.* **18**, 3992-4005.
McCracken, K. G., Bulgarella, M., Johnson, K. P., Kuhner, M. K., Trucco, J., Valqui, T. H., Wilson, R. E. and Peters, J. L. (2009b). Gene flow in the face of countervailing selection: adaptation to high-altitude hypoxia in the βA hemoglobin subunit of yellow-billed pintails in the andes. *Mol. Ecol. Evol.* **26**, 815-827.
Meir, J. U. and Ponganis, P. J. (2009). High-affinity hemoglobin and blood oxygen saturation in diving emperor penguins. *J. Exp. Biol.* **212**, 3330-3338.
Mineo, P. M., Cassell, E. A., Roberts, M. E. and Schaeffer, P. J. (2012). Chronic cold acclimation increases thermogenic capacity, non-shivering thermogenesis and muscle citrate synthase activity in both wild-type and brown adipose tissue deficient mice. *Comp. Biochem. Physiol. A Mol. Integr. Physiol.* **161**, 395-400.
Mirceta, S., Signore, A. V., Burns, J. M., Cossins, A. R., Campbell, K. L. and Berenbrink, M. (2013). Evolution of mammalian diving capacity traced by myoglobin net surface charge. *Science* **340**, 1234192.
Moore, L. G., Zamudio, S., Zhuang, J., Droma, T. and Shohet, R. V. (2002). Analysis of the myoglobin gene in Tibetans living at high altitude. *High Alt. Med. Biol.* **3**, 39-47.
Natarajan, C., Projecto-Garcia, J., Moriyama, H., Weber, R. E., Muñoz-Fuentes, V., Green, A. J., Kopuchian, C., Tubaro, P. L., Alza, L., Bulgarella, M. et al. (2015). Convergent Evolution of hemoglobin function in high-altitude andean waterfowl involves limited parallelism at the molecular sequence level. *PLoS Genet.* **11**, e1005681.
Pages, T. and Planas, J. (1983). Muscle myoglobin and flying habits in birds. *Comp. Biochem. Physiol. A Physiol.* **74**, 289-294.
Passarella, S., de Bari, L., Valenti, D., Pizzuto, R., Paventi, G. and Atlante, A. (2008). Mitochondria and l-lactate metabolism. *FEBS Lett.* **582**, 3569-3576.
Petit, M. and Vézina, F. (2014). Phenotype manipulations confirm the role of pectoral muscles and haematocrit in avian maximal thermogenic capacity. *J. Exp. Biol.* **217**, 824-830.
Petschow, D., Wurdinger, I., Baumann, R., Duhm, J., Braunitzer, G., & Bauer, C. (1977). Causes of high blood O2 affinity of animals living at high altitude. *J. App. Physiol.* **42**, 139-143.
Reed, J. Z., Butler, P. J. and Fedak, M. A. (1994). The metabolic characteristics of the locomotory muscles of grey seals (*Halichoerus grypus*), harbour seals (*Phoca vitulina*) and Antarctic fur seals (*Arctocephalus gazelle*). *J. Exp. Biol.* **194**, 33-46.
Reynafarje, B. (1962). Myoglobin content and enzymatic activity of muscle and altitude adaptation. *J. Appl. Physiol.* **17**, 301-305.
Reynafarje, B. (1963). Simplified method for the determination of myoglobin. *J. Lab. Clin. Med.* **61**, 138-145.
Reynafarje, B. and Morrison, P. (1962). Myoglobin levels in some tissues from wild Peruvian rodents native to high altitude. *J. Biol. Chem.* **237**, 2861-2864.
Rosser, B. W. C. and Hochachka, P. W. (1993). Metabolic capacity of muscle fibers from high-altitude natives. *Eur. J. Appl. Physiol.* **67**, 513-517.
Rubi, B., del Arco, A., Bartley, C., Satrustegui, J. and Maechler, P. (2004). The malate-aspartate NADH shuttle member Aralar1 determines glucose metabolic fate, mitochondrial activity, and insulin secretion in beta cells. *J. Biol. Chem.* **279**, 55659-55666.
Schantz, P. G. and Henriksson, J. (1987). Enzyme levels of the NADH shuttle systems: measurements in isolated muscle fibres from humans of differing physical activity. *Acta Physiol. Scand.* **129**, 505-515.
Schippers, M.-P., Ramirez, O., Arana, M., Pinedo-Bernal, P. and McClelland, G. B. (2012). Increase in carbohydrate utilization in high-altitude andean mice. *Cur. Biol.* **22**, 2350-2354.
Scott, G. R. and Milsom, W. K. (2006). Flying high: a theoretical analysis of the factors limiting exercise performance in birds at altitude. *Respir. Physiol. Neurobiol.* **154**, 284-301.
Scott, G. R. and Milsom, W. K. (2007). Control of breathing and adaptation to high altitude in the bar-headed goose. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **293**, R379-R391.
Scott, G. R., Cadena, V., Tattersall, G. J. and Milsom, W. K. (2008). Body temperature depression and peripheral heat loss accompany the metabolic and ventilatory responses to hypoxia in low and high altitude birds. *J. Exp. Biol.* **211**, 1326-1335.
Scott, G. R., Egginton, S., Richards, J. G. and Milsom, W. K. (2009a). Evolution of muscle phenotype for extreme high altitude flight in the bar-headed goose. *Proc. R. Soc. B Biol. Sci.* **276**, 3645-3653.
Scott, G. R., Richards, J. G. and Milsom, W. K. (2009b). Control of respiration in flight muscle from the high-altitude bar-headed goose and low-altitude birds. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **297**, R1066-R1074.
Scott, G. R., Schulte, P. M., Egginton, S., Scott, A. L. M., Richards, J. G. and Milsom, W. K. (2011). Molecular evolution of cytochrome c oxidase underlies high-altitude adaptation in the bar-headed goose. *Mol. Biol. Evol.* **28**, 351-363.
Scott, G. R., Hawkes, L. A., Frappell, P. B., Butler, P. J., Bishop, C. M. and Milsom, W. K. (2015a). How bar-headed geese fly over the Himalayas. *Physiology* **30**, 107-115.
Scott, G. R., Elogio, T. S., Lui, M. A., Storz, J. F. and Cheviron, Z. A. (2015b). Adaptive modifications of muscle phenotype in high-altitude deer mice are associated with evolved changes in gene regulation. *Mol. Biol. Evol.* **32**, 1962-1976.
Scrutton, M. C. and Utter, M. F. (1968). The regulation of glycolysis and gluconeogenesis in animal tissues. *Annu. Rev. Biochem.* **37**, 249-302.
Semenza, G. L., Roth, P. H., Fang, H. M., & Wang, G. L. (1994). Transcriptional regulation of genes encoding glycolytic enzymes by hypoxia-inducible factor 1. *J. Bio. Chem.* **269**, 23757-23763.
Shahriari, A., Dawson, N. J., Bell, R. A. V. and Storey, K. B. (2013). Stable suppression of lactate dehydrogenase activity in the foot muscle of *Littorina littorea* and the potential role of acetylation as a novel regulatory mechanism. *Enz. Res.* **2013**, 461374.
Sheafor, B. A. (2003). Metabolic enzyme activities across an altitudinal gradient: an examination of pikas (genus *Ochotona*). *J. Exp. Biol.* **206**, 1241-1249.
Simon, L. M., Robin, E. D., Elsner, R., Van Kessel, A. L. G. J. and Theodore, J. (1974). A biochemical basis for differences in maximal diving time in aquatic mammals. *Comp. Biochem. Physiol. B* **47**, 209-215.
Sirsat, S. K. G., Sirsat, T. S., Price, E. R. and Dzialowski, E. M. (2016). Post-hatching development of mitochondrial function, organ mass and metabolic rate in two ectotherms, the American alligator (*Alligator mississippiensis*) and the common snapping turtle (*Chelydra serpentina*). *Biol. Open* **5**, 443-451.
Snyder, G. K., Byers, R. L. and Kayar, S. R. (1984). Effects of hypoxia on tissue capillarity in geese. *Respir. Physiol.* **58**, 151-160.
Swan, L. W. (1970). Goose of the himalayas. *Nat. Hist.* **70**, 68-75.
Tamburrini, M., Condò, S. G., Di Prisco, G. and Giardina, B. (1994). Adaptation to extreme environments: structure-function relationships in emperor penguin haemoglobin. *J. Mol. Biol.* **237**, 615-621.

Telford, J. E., Kilbride, S. M. and Davey, G. P. (2009). Complex I is rate-limiting for oxygen consumption in the nerve terminal. *J. Biol. Chem.* **284**, 9109-9114.
Teulier, L., Rouanet, J.-L., Letexier, D., Romestaing, C., Belouze, M., Rey, B., Duchamp, C. and Roussel, D. (2010). Cold-acclimation-induced non-shivering thermogenesis in birds is associated with upregulation of avian UCP but not with innate uncoupling or altered ATP efficiency. *J. Exp. Biol.* **213**, 2476-2482.
Todd, F. S. (1996). White-water ducks: torrent duck. In *Natural History of the Waterfowl* (ed. F. S. Todd), pp. 245-260. Vista, Ca: Ibis Publishing Company.
Turner, D. L. and Butler, P. J. (1988). The aerobic capacity of locomotory muscles in the tufted duck, *Aythya fuligula*. *J. Exp. Biol.* **135**, 445-460.
Van Hall, G. (2000). Lactate as a fuel for mitochondrial respiration. *Acta Physiol. Scand.* **168**, 643-656.
Vaughan, B. E. and Pace, N. (1956). Changes in myoglobin content of the high altitude acclimatized rat. *Am. J. Physiol.* **185**, 549-556.
Ventura-Clapier, R., Kuznetsov, A., Veksler, V., Boehm, E. and Anflous, K. (1998). Functional coupling of creatine kinases in muscles: species and tissue specificity. *Mol. Cell. Biochem.* **184**, 231-247.
Vogt, A. M., Nef, H., Schaper, J., Poolman, M., Fell, D. A., Kübler, W. and Elsässer, A. (2002a). Metabolic control analysis of anaerobic glycolysis in human hibernating myocardium replaces traditional concepts of flux control. *FEBS Lett.* **517**, 245-250.
Vogt, A. M., Poolman, M., Ackermann, C., Yildiz, M., Schoels, W., Fell, D. A. and Kübler, W. (2002b). Regulation of Glycolytic Flux in Ischemic Preconditioning: a Study Employing Metabolic Control Analysis. *J. Biol. Chem.* **277**, 24411-24419.
Wastl, H. and Leiner, G. (1931). Observations on the blood gas in birds. I. Announcement. *Pflugers Arch.* **227**, 367-420.
Weibel, E. R. (1984). *The Pathway for Oxygen*. Cambridge, MA: Harvard University Press.
West, G. C. (1965). Shivering and heat production in wild birds. *Physiol. Zool.* **38**, 111-120.
Wittenberg, J. B. and Wittenberg, B. A. (2006). Myoglobin function reassessed. *J. Exp. Biol.* **206**, 2011-2020.
Wolfe, R. R. (1998). Metabolic interactions between glucose and fatty acids in humans. *Am. J. Clin. Nutr.* **67**, 519S-526S.
Wright, T. J. and Davis, R. W. (2015). Myoglobin oxygen affinity in aquatic and terrestrial birds and mammals. *J. Exp. Biol.* **218**, 2180-2189.
Xin, Y., Tang, X., Wang, H., Lu, S., Wang, Y., Zhang, Y. and Chen, Q. (2015). Functional characterization and expression analysis of myoglobin in high-altitude lizard *Phrynocephalus erythrurus*. *Comp. Biochem. Physiol. B Biochem. Mol. Biol.* **188**, 31-36.
Zhang, J., Hua, Z. Q., Tame, J. R. H., Lu, G. Y., Zhang, R. J. and Gu, X. C. (1996). The crystal structure of a high oxygen affinity species of haemoglobin (bar-headed goose haemoglobin in the oxy form). *J. Mol. Biol.* **255**, 484-493.