Flight muscle and heart phenotypes in the high-flying ruddy shelduck

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Abstract

Ruddy shelduck migrate from wintering grounds in lowland India and Myanmar to breeding grounds in central China and Mongolia, sustaining flight over the Himalayas, where oxygen availability is greatly reduced. We compared phenotypes of the pectoralis muscle and the ventricle of the heart from ruddy shelduck and common shelduck (a closely related low-altitude congener) that were raised in common conditions at sea level, predicting that oxidative capacity would be greater in ruddy shelduck to support high-altitude migration. Fibre-type composition of the pectoralis and the maximal activity of eight enzymes involved in mitochondrial energy metabolism in the pectoralis and heart, were compared between species. Few differences distinguished ruddy shelduck from common shelduck in the flight muscle, with the exception that ruddy shelduck had higher activities of complex II and higher ratios of complex IV (cytochrome c oxidase) and complex II when expressed relative to citrate synthase activity. There were no species differences in fibre-type composition, so these changes in enzyme activity may reflect an evolved modification in the functional properties of muscle mitochondria, potentially influencing mitochondrial respiratory capacity and/or oxygen affinity. Ruddy shelduck also had higher lactate dehydrogenase activity concurrent with lower pyruvate kinase and hexokinase activity in the left ventricle, which likely reflects an increased capacity for lactate oxidation by the heart. We conclude that changes in pathways of mitochondrial energy metabolism in the muscle and heart may contribute to the ability of ruddy shelduck to fly at high altitude.

Key words: Muscle phenotype, histology, enzyme, migrant, high altitude

Abbreviations

CII – Complex II

CIV – Complex IV (cytochrome c oxidase)

CS – Citrate synthase

HK – Hexokinase

HOAD – 3-hydroxyacyl-CoA dehydrogenase

IDH – Isocitrate dehydrogenase

LDH – Lactate dehydrogenase

PK – Pyruvate kinase

P_{CHEM} – Chemical power (W)

V_{MAX} - Maximal enzyme activity (μmol min^{-1} g^{-1} tissue)

V_{MP} - Minimum power speed (km hr^{-1})

Introduction

High altitude presents a physiological challenge to animal life as it comes with increased exposure, cold temperatures, and reduced barometric pressure resulting in diminished oxygen availability. Such conditions are inhospitable for many species (Prins and Namgail 2017), as animals must either reduce their demand for oxygen through a suppression of metabolic rate (i.e. through torpor) (Boutilier 2001) or make adjustments in the O_2 transport cascade to help maintain tissue oxygen utilization (McClelland and Scott 2018; Storz et al. 2019). Flapping flight, used by migrating waterfowl, is one of the most metabolically costly forms of locomotion per unit of time (Schmidt-Nielsen, 1971; Ward et al. 2001). Birds that migrate over high-altitude mountain ranges must overcome the challenge of meeting this high O_2 demand in conditions of low O_2 availability (Altshuler and Dudley 2006).

In order to support flight in hypoxic conditions there must be adequate delivery of oxygen to respiring cells, and then sufficient capacity for ATP production by mitochondria within active muscles (Butler 2010; Scott et al. 2015; Laguë 2017). Indeed, some high-altitude taxa have been shown to have increased oxidative capacity in the locomotory muscle (Dawson et al. 2016; Scott et al. 2018). For example, the high-altitude migrating bar-headed goose *(Anser indicus)* is able to fly from sea level in India to breeding grounds in Mongolia, migrating over the Himalayas and Tibetan Plateau (mean altitude ~ 4,500 m above sea level) whilst sustaining flight at up to 7,290 m altitude (Hawkes et al. 2012). This species has a significantly greater areal density of oxidative (principally type IIa) fibres (particularly in the superficial layers of pectoralis muscle) than lowland geese (Scott et al. 2009). Similarly, mitochondrial volume density is elevated in both the flight and leg muscles of grey crowned rosy finch (*Leucosticte arctoa*) at high altitude (Hepple et al. 1998; Mathieu-Costello et al. 1998).

The oxidative capacity of muscle is ultimately a result of the integrated function of the enzymes involved in mitochondrial energy metabolism, so the activity of metabolic enzymes can also be measured to gain insight into the oxidative capacity of muscles. For example, Dawson et al. (2016) sampled locomotory and cardiac muscles in torrent duck (*Merganetta armata*), a riverine species that swims in fast-flowing mountain rivers using leg muscles, in populations resident at either high (>3,000 m) or low (<1,665 m) altitudes in the Andes. High-altitude residents had higher mitochondrial oxidative capacity in the gastrocnemius muscle of the leg muscle (measured as the respiratory capacity of permeabilized muscle fibres) in association with higher activity of cytochrome c oxidase, the terminal O_2 acceptor in the electron transport system. However, changes in enzyme activity have not been observed in some other high-altitude bird taxa (e.g. Andean coot, *Fulica ardesiaca*) (León-Velarde et al.

1993). Otherwise, there has been relatively little work on other high-altitude birds, particularly those that complete a high-altitude migration (with the notable exception of the bar-headed goose).

The ruddy shelduck *(Tadorna ferruginea)* has a broad distribution across Eurasia but in the core of their distribution, migrate annually from wintering grounds in southern India and Myanmar to breeding grounds in central China and Mongolia, sustaining flights at altitudes up to 6,800 m and climbing up to 0.45 metres per second during their migration across the Himalayas (Parr et al. 2017). This migration, akin to that of the bar-headed goose, suggest that ruddy shelduck may require physiological adaptations to support migration in hypobaric hypoxia. Thus, in the present study, we investigated flight muscle and heart phenotypes of ruddy shelduck, comparing them with a congeneric species raised in common garden conditions at sea level, the common shelduck (*Tadorna tadorna*), which live year round at comparatively low altitudes, with migratory and resident populations across central Europe, northern Africa and eastern Asia (Hoyo et al. 1992). We measured several indices of oxidative capacity (fibre-type composition and metabolic enzyme activities), and predicted that ruddy shelduck would have (i) a higher proportion of oxidative fibres in flight muscle, and (ii) higher activities of oxidative enzymes in both flight and cardiac muscle to facilitate high-altitude migration.

Materials and Methods

Experimental Animals

Ruddy shelduck (0.75 to 1.4 kg, n= 16) and common shelduck (0.70 to 1.00 kg, n= 16) were obtained as young adults (1 to 2 years) of both sexes and kept in captivity, having been bred at sea level by registered commercial breeders in the UK. The birds had thus never flown or been exposed to high altitude. Eight birds from each species were sampled for enzyme analyses in April 2017 and eight birds from each species were sampled for histological analyses in October 2018.

In order to take samples from the pectoralis major (the major muscle that powers the downstroke of the wing during flight) and the left ventricle of the heart, the birds were euthanised with an intravenous overdose of euthatal (pentobarbital), and death was confirmed with cervical dislocation. A slice of the left pectoralis, approximately 1 cm thick through the entire depth of the muscle and parallel to the oriententation of the muscle fibres, was quickly dissected out. From this slice, samples (~1 cm^3) were taken near the middle of the muscle at each of three depths: (i) superficial depth at the subcutaneous surface, (ii) intermediate depth, and (iii) deep samples nearest the sternum. The bird's right pectoralis major was then dissected out to be weighed. The heart was then dissected out, the ventricles were weighed after the atria was trimmed away, and samples were taken from the left ventricle that included the full thickness of the ventricle wall. Samples from both pectoralis and heart for enzyme analyses were immediately frozen in liquid N_2 and stored at -80°C. Samples from pectoralis for histology were prepared as follows: the 1cm^3 sample was coated in mounting medium (Sigma-Aldrich M1289), flash frozen in liquid N_2-cooled 2-methylbutane, and stored at -80°C until processing. Sampling took place at Fera Science Limited, York, and at the University of Exeter, UK, with protocols approved by the University of Exeter's ethical committee.

Flight muscle histology

Following previously published protocols (Scott et al., 2009), each of the histology samples were sectioned (10µm) transverse to fibre length in a -20°C cryostat. Sections were stained for myosin-ATPase activity to identify type *I*, type *IIa* and type *IIb* fibres following an acidic pre-incubation for six minutes at pH 4.3. All sections were imaged using light microscopy, systematically taking as many images as possible without duplication. Following imaging, image J software (version 1.49, Schneider et al., 2012) was used to make unbiased measurements of the numerical and areal densities and the average transverse areas of each fibre type. Preliminary analyses determined

that a stable mean value of each fibre type could be achieved by analyzing 15 images per bird across the three sampling depths.

Enzyme activity assays

Following previously published protocols (Dawson et al. 2016), samples were weighed and homogenized on ice in 10 volumes of homogenisation buffer A (100 mmol l^{-1} KH_2PO_4 buffer, pH 7.2, containing 1 mmol l^{-1} EGTA, 1 mmol l^{-1} EDTA, 1 mmol l^{-1} phenylmethylsulfonyl fluoride (PMSF) and 0.1% Triton-X) using a glass Tenbroeck tissue grinder. Homogenate was then centrifuged at 1000g for 1 minute at 4°C and the supernatant collected and placed on ice for the enzyme assays. Eight enzymes (detailed below) were assayed in triplicate for maximal activity (V_{MAX}) in each sample. V_{MAX} was measured as the mean reaction rate in the presence of all substrates (at saturating concentrations), minus the background reaction rate (determined by the absence of a key substrate ("-")). The only exception was complex IV, for which the background reaction rate was expected to be very low and was not measured. In a small number of samples, we did not obtain reliable activity measurements with good technical replication. These samples were excluded from the dataset, and the resulting sample sizes for each enzyme are stated in Fig. 2 and Fig. 3.

Measurements were carried out at 41 °C in 100 mmol l^{-1} KH_2PO_4 buffer (pH 7.2) under the following assay conditions once preliminary experiments had determined that all substrate concentrations were saturating. Complex II [CII; ε=21.9 l mmol l^{-1} cm^{-1} at 600 nm]: 20 mmol l^{-1} succinate (−), 0.3 mmol l^{-1} KCN, 0.05 mmol l^{-1} dichlorophenolindophenol, 0.05 mmol l^{-1} decylubiquinone. Complex IV [CIV; ε=28.5 l mmol l^{-1} cm^{-1} at 550 nm]: 0.2 mmol l^{-1} reduced cytochrome c. Hexokinase [HK; ε=6.22 l mmol l^{-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. Pyruvate kinase [PK; ε=6.22 l mmol l^{-1} cm^{-1} at 340nm)]: 10 mmol l^{-1} phosphoenolpyruvate (−), 2.5 mmol l^{-1} Mg·ADP, 10 mmol l^{-1} $MgCl_2$, 0.3 mmol l^{-1} NADH, 1 unit of lactate dehydrogenase. Lactate dehydrogenase [LDH; ε=6.22 l mmol l^{-1} cm^{-1} at 340 nm]: 5 mmol l^{-1} pyruvate (−), 0.3 mmol l^{-1} NADH. Citrate synthase [CS; ε=14.15 l mmol l^{-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. Isocitrate dehydrogenase [IDH; ε=6.22 l mmol l^{-1} cm^{-1} at 340 nm]: 5 mmol l^{-1} isocitrate (−), 1.5 mmol l^{-1} $NADP^+$. 3-hydroxyacyl-CoA dehydrogenase [HOAD; ε=6.22 l mmol l^{-1} cm^{-1} at 340 nm]: 0.15 mmol l^{-1} acetoacetyl CoA (−), 0.3 mmol l^{-1} NADH.

Assays were measured using a SpectraMax Plus 384 spectrophotometer (Molecular Devices, Sunnyvale, CA, USA) and data were analyzed using the accompanying SoftMax Pro 6.3 program and R (R Core Team 2017).

V_{MAX} was expressed per gram of tissue per minute. Protein concentrations were also determined using the BCA method with bovine serum albumin as a standard (following instructions from Sigma-Aldrich).

Tracking data

To investigate the approximate flight costs shelduck may incur during their migration, we estimated the minimum power speed (V_{MP}) and chemical power (P_{CHEM}) required for ruddy shelduck to undertake level flight, using Flight 1.25 (Pennycuick 2008). In the absence of data describing wild pre-migratory bird masses, we used 1.2 kg body mass, 1.3 m wing span (Carboneras and Kirwan 2020) and 0.21 m^2 wing area (to give an aspect ratio of 8.1, the mean aspect ratio for ruddy shelduck in this study). We also estimated V_{MP} and P_{CHEM} for a 2.8 kg bar-headed goose using a 1.46 m wing span and 0.252 m^2 wing area (Lee et al., 2008; Bishop et al., 2015). Finally, we then converted estimates of chemical power (W) to oxygen consumption using the assumption that 20.9 J equates to 1 mL O_2.

Statistical analysis

All analysis and plotting was completed using R (R Core Team 2017). Because ruddy shelduck (mean mass 1.039 kg ± 0.17 s.d.) are larger than common shelduck (0.83 kg ± 0.09) we initially included body mass and organ mass in the histology analysis to control for any affect on fibre size or fibre composition. However, neither body mass nor pectoral mass explained significant variation in these response variables and were removed from all histological analyses relating to fiber type or size. Linear regression was used to compare differences between species in average fibre size, with species as the only predictor. A general linear model was used to compare muscle-fibre composition (with a quasibinomial distribution as the data is limited between 0 and 1) with species as the predictor. Model selection was done using likelihood ratio tests.

Linear models were used to investigate if maximal activity varied by species for each enzyme. The response variable was the maximal enzyme activity (μmol min^{-1} g^{-1} tissue) with body mass and species as predictors. Sampling depth and the interaction between species and sampling depth were also included in the analysis of flight muscle samples. To examine the effect of species in the maximal activity of enzymes in the electron transport system relative to CS activity (a marker of mitochondrial volume), we calculated the ratio of V_{MAX} for CII and CIV to CS for each sample and then assessed for the effect of species with linear models. In these models, the calculated activity ratios were the response variables, with body mass, species, sampling depth and the interaction between species and sampling depth as predictors. We carried out stepwise reduction using likelihood

ratio tests to remove non-significant predictors from all models and present the full and reduced models (Table S1).

Results

Shelduck morphology

Because ruddy shelduck body mass is 25% greater than common shelduck (*linear model*; $t_{(1,29)}$ =6.891, P≤0.001, Table 1) after controlling for sex (*linear model;* $t_{(1,29)}$ =6.829, P≤0.001), organ size comparisons were carried out relative to body size (with a quasibinomial distribution). Relative pectoral mass was greater in ruddy shelduck (*general linear model*; $t_{(1,30)}$ =2.369, P=0.025, Table 1), however, there were no significant differences between ruddy and common shelduck in relative ventricle mass (*general linear model*; $t_{(1,29)}$=0.203, P=0.84), or relative lung mass (*general linear model*; $t_{(1,29)}$=1.289 , P=0.207, Table 1).

Flight muscle histology

There was a significant difference in fast oxidative type *II*a fibres according to the method used to measure density; mean areal density (0.834 ± 0.05 s.d.) of fast oxidative type *II*a fibres was lower than mean numeric density (0.905 ± 0.03, *general linear model*; $t_{(30)}$ = 4.486, P≤0.001) across species. This modest difference is expected due to the smaller size of type IIa fibres compared to IIb fibres (Table 1). The areal density method provides a better overall estimate of the proportion of total muscle occupied by each fibre type, as it accounts for fibre size (Egginton 1990), so areal density was used to evaluate differences in the total muscle composition between the two species from hereon, though we note the results were not changed when using numeric densities. We highlight this important methodological consideration for comparative work.

In both the common and ruddy shelduck, the pectoralis muscle was predominantly composed of type *IIa* fast oxidative fibres (mean 82.1% for ruddy shelduck and 84.7 % for common shelduck; Table 1, Fig. 1A, 1B). The remaining minority of fibres were type *IIb* fast glycolytic fibres (mean 17.9% for ruddy shelduck and 15.3% for common shelduck; Fig. 1A). The total proportion of oxidative fibres across the pectoral muscle did not differ between species (*general linear model;* $t_{(1,14)}$=1.007, P=0.331, Table 1). Staining did not identify any type *I* slow oxidative fibres, which have acid-insensitive myosin-ATPase and would therefore exhibit a characteristic dark black staining pattern that was not observed (Fig. 1A).

Type *IIb* fast glycolytic fibres were approximately two-fold larger than oxidative fibres across both species, but fibre size was not affected by species for either fibre type; ruddy shelduck fast glycolytic type *II*b mean fibre size was 2045.167 μm^2 ± 301.57 s.d. compared to 1817.792 μm^2 ± 511.28 in common shelduck (*linear model*, $t_{(1,14)}$=1.083, P=0.297). Likewise, ruddy shelduck fast oxidative type *II*a fibre size (997.727 μm^2 ± 126.97) did not differ from common shelduck (922.743 μm^2 ± 221.59, *linear model*, $t_{(1,14)}$=0.83, P=0.420).

Enzyme activities in flight muscle

Within the flight muscle CII maximal activity (V_{MAX}) was significantly affected by species (*linear model*, $F_{(1,39)}$=8.204, P=0.007, Table S1), with greater activity in ruddy shelduck than common shelduck (Fig 2, Table 2). However, V_{MAX} across all other enzymes were very similar between species (Table 2, Fig. 2). In general, V_{MAX} varied with sampling depth for all enzymes (Table S1, Fig. 2). The activities of enzymes involved in mitochondrial electron transport (CII), the tricarboxylic acid cycle (CS), and lipid oxidation (HOAD) increased with depth, suggesting a higher oxidative capacity, whereas the activities of some glycolytic enzymes (LDH, PK) decreased with depth (Fig. 2). There was a significant interaction between species and sampling depth for HK, such that HK was significantly higher in common shelduck at the intermediate sampling level (by 39%, Tukey HSD, P=0.001). Protein content of the flight muscle was significantly greater in the ruddy shelduck in comparison to the common shelduck after controlling for depth (*linear model*, $t_{(1,44)}$=2.02, P=0.049, Table 1), however this was found to be a non-significant predictor when evaluated in preliminary linear modelling for all enzymes in the flight muscle.

HK was also found to be lower in ruddy shelduck cardiac muscle (by 24.7%, *linear model*, $F_{(1,13)}$=5.36, P=0.038, Fig.3, Table S1). In addition, enzymes involved in the metabolism of carbohydrates differed between shelduck species in the cardiac muscle; LDH was 39.1% greater in ruddy shelduck than common shelduck (*linear model*, $F_{(1,14)}$=32.392, P=<0.001), whereas PK was 27.8% lower in ruddy shelduck (*linear model*, $F_{(1,13)}$=8.528, P=0.012, Table 2, Table S1, Fig. 3).

We also examined the activity ratios between enzymes in the electron transport chain relative to CS (a common marker of mitochondrial volume density) to assess how the functional properties of mitochondria might be altered in ruddy shelduck. The ratio of CII to CS activity in ruddy shelduck flight muscle (0.13) was higher than common shelduck (0.07, *linear model with a square root transformation*, $F_{(1,39)}$=15.4, P≤0.001, Fig. 4). This was also found for the ratio of CIV to CS (0.23 in ruddy shelduck, 0.20 in common shelduck, *linear model with a square root transformation*, $F_{(1,43)}$=5.167, P=0.030, Fig. 4). In contrast, no differences in activity ratios were found between species in the left ventricle.

Tracking data

Because ruddy shelduck have a lower body mass than bar-headed geese, ruddy shelduck minimum power speeds (V_{MP}) are lower, at 44.3 km hr^{-1} versus 55.4 km hr^{-1} for bar-headed geese, at sea level. Consequently, flight costs for ruddy shelduck may be reduced, thus, to fly at sea level a ruddy shelduck requires less oxygen than a bar-headed goose (an estimated 108 versus 150 ml O_2 min^{-1} kg^{-1}). For both species, flight costs increase from sea level to 4,500 metres (approximate height of the Tibetan Plateau) by approximately 25% because the reduction in air density requires faster forward speeds to remain aloft (Pennycuick 2008). However, given the lower V_{MP} of ruddy shelduck, in absolute terms the oxygen demand for ruddy shelduck is approximately 72% that of bar-headed geese when flying at 4,500 m.

Discussion

In the present study we examined differences in metabolic enzyme activity between ruddy shelduck, a high-altitude migrating duck that can fly up to 6,800 metres across the eastern Himalayas (Parr et al. 2017), and common shelduck. In the flight muscle, complex II had higher activities in ruddy shelduck. In addition, activity ratios of mitochondrial electron transport system complexes II and IV relative to CS (a common marker of mitochondrial volume density) were higher in ruddy shelduck, without any species differences in fibre-type composition, suggesting that there are evolved changes in the functional properties (rather than the abundance) of mitochondria. In the left ventricle, LDH activity was higher but PK and HK activity were lower in ruddy shelduck, suggesting that this species may possess an enhanced capacity for lactate oxidation. These unique features of ruddy shelduck were observed in comparisons in common conditions at sea level and may therefore represent evolved specializations in mitochondrial function and energy metabolism that may be associated with high-altitude flight.

Species differences in enzyme activity may reflect changes in mitochondrial function

The greatest variation in flight muscle maximal enzyme activity between ruddy and common shelduck were observed within the electron transport chain. CII had a higher V_{MAX} in ruddy shelduck flight muscle relative to common shelduck, both in absolute terms and when expressed relative to CS activity. Complex IV (CIV, cytochrome c oxidase) also had higher activities in ruddy shelduck flight muscle relative to common shelduck, when expressed relative to CS activity. The latter may contribute to an increase in the relative excess of CIV in the muscle mitochondria in ruddy shelduck. An excess of CIV has been suggested to enhance the oxygen binding affinity of mitochondria, by increasing the availability of oxygen binding sites and reducing the catalytic turnover of each CIV enzyme, which would help mitochondria maintain ATP synthesis at lower tissue oxygen levels (Ou and Tenney 1970; Gnaiger et al. 1998). CIV activity is higher (without a change in CS activity) in high-altitude populations of torrent ducks relative to low-altitude populations when sampled from the gastrocnemius muscle in the leg (Dawson et al. 2016). This is a more appropriate muscle for comparison with shelduck than flight muscle, given that swimming (rather than flying) is the primary mode of locomotion for the torrent duck (Johnsgard 1978). Furthermore, in the high-altitude torrent ducks, CIV was the only enzyme in the electron transport system whose activity was associated with enhanced respiratory capacity (Dawson et al. 2016). Taken together, higher CIV activity relative to CS in ruddy shelduck and higher absolute CIV activity in torrent duck may suggest that changes in mitochondrial function are a common part of high-altitude adaptation.

Some enzymes involved in carbohydrate metabolism differed between ruddy and common shelduck in the cardiac muscle. LDH in particular was at considerably higher activity in the cardiac muscle of ruddy shelduck than common shelduck. Dawson et al. (2016) also showed that LDH maximal activity was elevated in cardiac muscle of high-altitude populations of torrent duck, suggesting that this may be a common response to high altitude across avian species. This increase in LDH activity could increase the capacity for using anaerobic metabolism during high-altitude hypoxia, but we believe this possibility is unlikely in ruddy shelduck for two reasons. Firstly, migratory flights are long, even at high altitude, and should thus be supported by aerobic metabolism (Parr et al. 2017). Secondly, PK and HK activities were significantly reduced in the cardiac muscle of ruddy shelduck, suggesting that glycolytic capacity is not augmented in the heart of this species. The increased LDH activity in ruddy shelduck may instead be important for supporting the oxidation of lactate in the heart, consuming any lactate produced by other tissues and contributing to the intercellular lactate shuttle (Brooks et al. 1999; Gladden 2004). Lactate may thus be an additional metabolic fuel source to support heart function during flight in high-altitude hypoxia. We did not observe any variation in enzymes involved in beta oxidation (i.e., HOAD) in ruddy shelduck in the cardiac muscle, in contrast to some other high-altitude natives (Scott et al. 2011; Dawson et al. 2016).

The activities of enzymes involved in carbohydrate metabolism were not generally altered in the flight muscle of ruddy shelduck. This contrasts some mammals at high altitude, in which the capacity for carbohydrate oxidation is enhanced relative to low altitude, potentially to take advantage of the relatively higher ATP/O_2 yield of carbohydrates compared to lipid (Schippers et al. 2012; Lau et al. 2017). This distinction may arise because fuel selection in the flight muscle of migratory birds may be under much greater constraint to metabolise fatty acids (the capacity for which is indicated by HOAD activity). Indeed, wild populations of shelduck may be predicted to have higher HOAD activity during the migratory period than in the captive ducks of the present study (Bishop et al. 1995). Fat is the metabolic fuel of choice in migrants (Guglielmo 2010; Butler 2016), likely because of the presumably immense selective pressure to minimize body weight for flight (far more ATP can be synthesised per gram from fat than from carbohydrates) (Kuzmiak-Glancy & Willis 2014). The capacity for beta oxidation of lipids could be elevated in the flight muscle of wild ruddy shelduck leading up to and during migration, but based on our observation here that HOAD activity was similar between species, there do not appear to be any constitutive changes in beta oxidation in this species.

Species differences in enzyme activity in the flight muscle were not caused by variation in fibre-type composition

289 Fibre-type composition has a strong influence on the activities of metabolic enzymes in skeletal muscles (Bass et
290 al. 1969), but this was not the cause of the species differences in CII activity or CIV activity relative to CS, as
291 fibre-type composition of the flight muscle was the same between ruddy shelduck and common shelduck. The
292 relative proportions of muscle fibre types in shelduck in the present study are similar to previous studies of some
293 other species of waterfowl, including bar-headed geese, pink-footed geese and barnacle geese – there was a
294 majority of type *IIa* fibres, with a minority of type *IIb* fibres and no type *I* fibres (Rosser and George 1986;
295 Lundgren and Kiessling 1988; Scott et al. 2009). However, contrary to our predictions, our findings differ from
296 previous studies of other high-altitude taxa (León-Velarde et al. 1993; Mathieu-Costello et al. 1998; Scott et al.
297 2009, 2011), in which there were higher densities of oxidative fibres in the flight muscle relative to low-altitude
298 groups.

299 Biomechanical modelling suggests that flight costs for ruddy shelduck may be 72% that of the costs for bar-headed
300 geese, flying at the maximum 6,800 metres that ruddy shelduck have been directly tracked (Parr et al. 2017).
301 Although the relative increase in oxygen demand increases at the same rate in both species, the absolute amount
302 of oxygen required for forward flight at V_{MP} in still air is theoretically lower for ruddy shelduck than for bar-
303 headed geese, due to the ruddy shelduck's lower body mass. This simulation of estimated flight costs suggests
304 that ruddy shelduck may be under a lesser selection pressure for physiological changes due to lower flight costs
305 than bar-headed geese, and may help explain why ruddy shelduck do not exhibit some of the changes in flight
306 muscle phenotype that are exhibited by bar-headed geese (e.g., increases in oxidative fibre-types in the flight
307 muscle).

308 However, despite the potentially weaker selection for physiological adaptation in ruddy shelduck relative to bar-
309 headed geese, it is possible that wild populations of shelduck show greater variation than the captive individuals
310 in the present study in both the histology of flight muscle and in maximal enzyme activity. Only one of the
311 previous studies that made comparisons of flight muscle phenotype between highland versus lowland taxa
312 controlled for environmental conditions (Scott et al. 2009). In studies that compared taxa in their native
313 environment (Dawson et al. 2016), phenotypic plasticity could have played very important roles in creating the
314 muscle phenotype at high altitude. Phenotypic and developmental plasticity almost certainly interacts with genetic
315 variation, environment, and flight demands to shape exercise capacity and its underlying determinants as well as
316 response to hypoxia (Storz et al. 2010; Piersma and van Gils 2011; McWilliams and Karasov 2014; Laguë et al.

2016). Therefore, muscle plasticity could be a very important contributor to the ability of ruddy shelducks to fly at high altitude.

Furthermore, the capacity for maximal activity of each respiratory enzyme is flexible and may respond to activity, hypoxia, cold temperature, or a combination of each. For example in torrent ducks, enzyme activity in the active leg muscle and cardiac samples were found to be much more variable between altitude groups than enzymes sampled from the comparatively less active flight muscle (Dawson et al. 2016), suggesting that only by using the muscle in hypoxic conditions had the maximal activity of certain enzymes become greater in the high-altitude populations. Furthermore, time of year has been shown to influence metabolic activity for migrants; birds up-regulate enzymes and haematocrit leading up to migration, relative to during breeding or wing moult (Bishop et al. 1995; McKechnie 2008; Krause et al. 2015; Fudickar et al. 2016), even in captive species (Portugal et al. 2007, 2009). It is thus likely that maximal levels of enzyme activity may vary throughout the year, in response to season (Dawson et al. 1983) or environmental temperature (Vézina et al. 2017). Furthermore, greater plasticity to respond to such stimuli may also evolve in high-altitude migrants (Storz et al. 2010). This suggests it is possible that the time of year in which shelduck were sampled in the present study, as well as their captivity (i.e. they could not fly) may directly influence the activity of enzymes and the muscle fibre-type composition. Therefore, differences between ruddy and common shelduck may be greater in wild populations throughout or at key times in the annual cycle. Nevertheless, the lack of variation in fibre-type composition observed here suggest that the species differences may reflect evolved changes in the mitochondrial electron transport system that may contribute to high-altitude flight.

Future research should aim to study wild populations of ruddy shelduck throughout their annual cycle to investigate whether developmental plasticity, described in other high-altitude migrants (Laguë et al. 2016), may confer a more oxidative phenotype that is not present in shelduck at low-altitude. Furthermore, avian long-distance migrants undergo a series of morphological, physiological and behavioural changes that increase the availability of metabolic substrates and muscular aerobic capacity in preparation for, and during, migration (Piersma and Drent 2003; Yap et al. 2017). Therefore, wild populations of migrating shelduck could have higher proportions of oxidative fibres and higher rates of oxidative enzyme activity, which may be further increased by exposure to the high-altitude environment experienced by ruddy shelduck in the wild.

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351

Metric	N	Common Shelduck	Ruddy Shelduck
Body Mass (kg)*	32	0.83 ± 0.09	1.039 ± 0.17
Pectoral Mass (% of body mass)*	32	7.9 ± 0.8	8.6 ± 0.8
Lung Mass (% of body mass)	32	0.9 ± 0.3	0.8 ± 0.3
Ventricle Mass (% of body mass)	32	0.6 ± 0.0	0.6 ± 0.1
Ventricle Tissue protein (mg g^{-1})	16	95.19 ± 20.78	94.06 ± 13.43
Pectoral Tissue protein (mg g^{-1})*	16	141.34 ± 13.97	156.15 ± 9.29
Type IIa Areal Density (proportion)	16	0.847 ± 0.05	0.821 ± 0.06
Type IIa Numeric Density (proportion)	16	0.912 ± 0.03	0.897 ± 0.04
IIa fibre transverse area (μm^2)	16	922.743 ± 221.59	997.727 ± 126.97
IIb fibre transverse area (μm^2)	16	1817.792 ± 511.28	2045.167 ± 301.57

Table 1 Muscle metrics and histological measurements from the flight muscle of shelduck. Data shown as mean ± s.d., N shows total across species, relative masses expressed as percentage of body mass (g). Asterisks indicates that there was a significant difference between species (P<=0.05)

Enzyme	Muscle	Common Shelduck	Ruddy Shelduck	Difference (%)
CII	Flight*	5.406 ± 2.42	8.033 ± 1.73	48.59
	Ventricle	3.281 ± 0.86	2.43 ± 1.18	-25.94
CIV	Flight	13.045 ± 0.55	13.955 ± 1.33	6.98
	Ventricle	9.751 ± 1.77	10.306 ± 1.89	5.69
CS	Flight	71.273 ± 6.85	64.209 ± 9.99	-9.91
	Ventricle	89.579 ± 15.41	76.415 ± 8.1	-14.7
HK	Flight	3.091 ± 0.58	2.33 ± 0.31	-24.62
	Ventricle*	1.353 ± 0.11	1.019 ± 0.37	-24.69
HOAD	Flight	29.501 ± 2.94	31.512 ± 4.51	6.82
	Ventricle	35.676 ± 3.92	33.394 ± 4.15	-6.4
IDH	Flight	17.811 ± 1.43	17.741 ± 1.35	-0.39
	Ventricle	0.776 ± 0.12	0.632 ± 0.13	-18.56
LDH	Flight	312.495 ± 26.71	304.813 ± 25.63	-2.46
	Ventricle*	91.791 ± 9.97	127.719 ± 14.81	39.14
PK	Flight	411.949 ± 53	433.164 ± 19.66	5.15
	Ventricle*	165.911 ± 15.85	119.831 ± 41.48	-27.77

Table 2 Summary of maximal enzyme activity in flight and ventricle muscles of shelduck.

Mean ± s.d. for enzyme V_{max} (μmol min^{-1} g^{-1} tissue) in the ventricle and pectoral samples and the difference of ruddy shelduck relative to common shelduck. *Indicates a significant difference (Table S1).

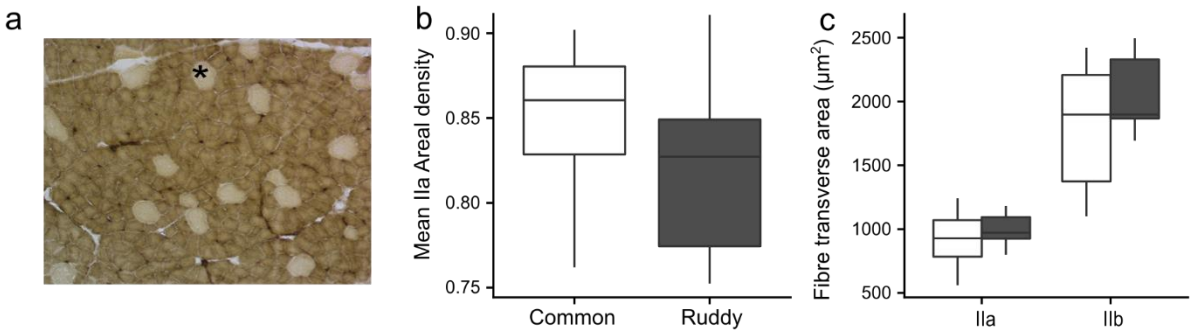


Fig 1 Flight muscle phenotype of ruddy and common shelduck. (a) ATPase activity staining in the pectoralis major muscle of shelduck (muscle samples were predominantly type *IIa* fast oxidative fibres with a lower proportion of type *IIb* fast glycolytic fibres (beige areas, example shown as asterisk). Ruddy shelduck are shown in grey (n=8), common shelduck shown in white (n=8). Boxplots show variation in (b) mean areal density of type IIa fibres and (c) IIa and IIb fibre size across the two species

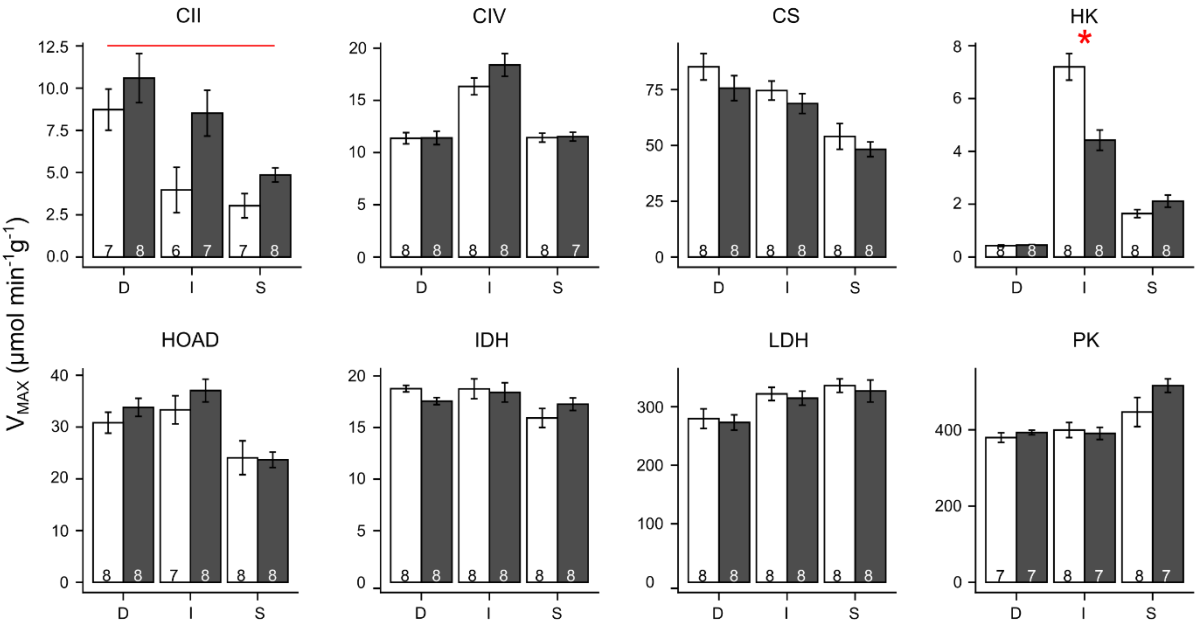
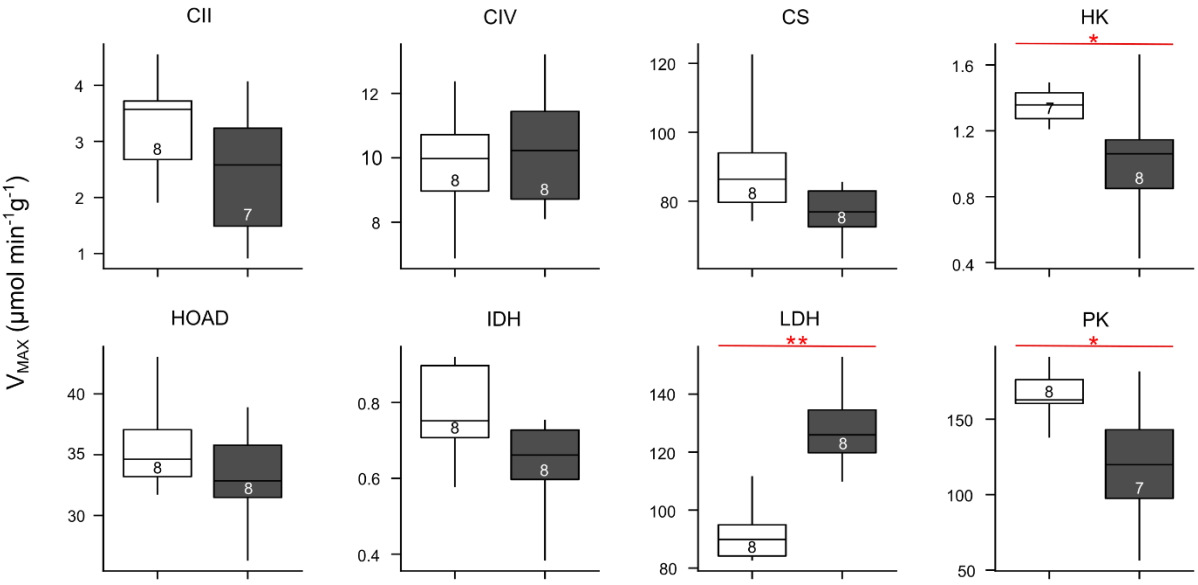


Fig 2 Maximal enzyme activity in flight muscle of ruddy and common shelduck. Mean V_{max} ± SE for enzymes sampled from the pectoralis major of common shelduck (white filled bars) and ruddy shelduck (grey filled bars), plotted at each muscle depth (surface (S), intermediate (I) and deep (D)). Sample size shown for each species and depth in bars. Red asterisk indicates significant difference between species at specific sampling depths ($P < 0.05$, Tukey Post Hoc testing), red line indicates a significant difference between species ($P<0.05$, Table S1)



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374 **Fig 3** Maximal enzyme activity in cardiac muscle of ruddy and common shelduck. Boxplots to show V_{max} activity
375 for enzymes in ventricle samples of common shelduck (white) and ruddy shelduck (grey). Sample size shown for
376 each species in boxplots. Red line indicates significant differences between species (single asterisk $P < 0.05$,
377 double asterisk $P \leq 0.001$, Table S1)

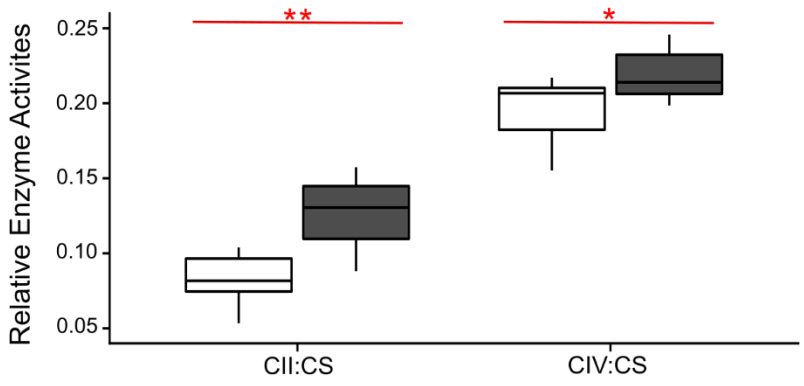

Fig 4 Enzyme activity ratios in ruddy and common shelduck. Boxplots to show the enzyme activity (µmol min^{-1} g^{-1}) in ruddy shelduck (grey) and common shelduck (white) of CII and CIV relative to CS activity in flight muscle. Red line indicates significant difference between species (single asterisk $P < 0.05$, double asterisk $P \leq 0.001$, Table S1)

					Body Mass		Species		Depth		Interaction	
Enzyme	Model	Predictors included in model	DF	R^2	F	P	F	P	F	P	F	P
Flight muscle samples												
CII	Full	Body + Species * Depth	36	0.504	0.587	0.449	0.577	0.452	6.894	0.003	0.828	0.445
	Reduced	Species + Depth	39	0.472			8.204	0.007	13.350	<0.001		
CIV (logged)	Full	Body + Species * Depth	40	0.699	0.559	0.459	0.087	0.770	17.132	<0.001	0.773	0.468
	Reduced	Depth	44	0.676					45.866	<0.001		
CS	Full	Body + Species * Depth	41	0.487	0.057	0.813	1.323	0.257	9.911	<0.001	0.093	0.912
	Reduced	Depth	45	0.447					18.161	<0.001		
HK (logged)	Full	Body + Species * Depth	41	0.960	2.788	0.103	1.168	0.286	297.871	<0.001	11.385	<0.001
	Reduced	Species * Depth	42	0.958			0.220	0.641	285.710	<0.001	10.920	<0.001
HOAD	Full	Body + Species * Depth	40	0.440	2.047	0.160	0.098	0.756	4.410	0.019	0.451	0.640
	Reduced	DEPTH	44	0.382					13.575	<0.001		
IDH	Full	Body + Species * Depth	41	0.225	0.731	0.398	1.943	0.171	4.933	0.012	1.536	0.227
	Reduced	Depth	45	0.153					4.068	0.024		
LDH	Full	Body + Species * Depth	41	0.305	1.046	0.312	0.477	0.494	4.295	0.020	0.005	0.995
	Reduced	Depth	45	0.279					8.724	0.001		
PK	Full	Body + Species * Depth	37	0.421	0.426	0.518	0.365	0.549	2.453	0.100	1.569	0.222
	Reduced	Depth	41	0.333					10.241	<0.001		
Flight ratio models												
CII/CS (square root)	Full	Body + Species * Depth	36	0.412	0.741	0.395	0.975	0.330	3.410	0.044	1.186	0.317
	Reduced	Species + Depth	39	0.360			15.362	<0.001	3.297	0.048		
CIV/CS (square root)	Full	Body + Species * Depth	40	0.549	0.040	0.842	0.680	0.415	9.070	<0.001	0.299	0.743
	Reduced	Species + Depth	43	0.542			5.167	0.030	23.075	<0.001		
Ventricle samples												
CII	Full	Body + Species	12	0.300	2.277	0.157	0.255	0.623				
	Reduced	Body	13	0.285	5.174	0.041						
CIV	Full	Body + Species	13	0.051	0.350	0.564	0.684	0.423				
	Reduced	~1	15	0.000								
CS	Full	Body + Species	13	0.324	1.501	0.242	1.114	0.310				
	Reduced	Body	14	0.266	5.084	0.041						
HK	Full	Body + Species	12	0.295	0.044	0.838	2.684	0.127				
	Reduced	Species	13	0.292			5.360	0.038				

HOAD	Full	Body + Species	13	0.246	2.789	0.119	0.000	0.998
	Reduced	~1	15	0.000				
IDH	Full	Body + Species	13	0.447	3.921	0.069	0.873	0.367
	Reduced	Body	14	0.410	9.733	0.008		
LDH	Full	Body + Species	13	0.711	0.571	0.463	24.950	<0.001
	Reduced	Species	14	0.698			32.392	<0.001
PK	Full	Body + Species	12	0.397	0.018	0.896	5.237	0.041
	Reduced	Species	13	0.396			8.528	0.012

Table S1 Test statistics for maximal enzyme activity in flight and ventricle muscles of shelduck

Linear regression F and P values for each enzyme maximal activity (μmol min^{-1} g^{-1} tissue) in the ventricle and pectoral samples, for full models and models following stepwise reduction to remove non-significant predictors. Predictors include body (continuous; body mass (g)), species (categorical; ruddy or common shelduck) and depth (categorical; surface, intermediate or deep sampling depth) and the interaction between species and depth.

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