Evolution of muscle phenotype for extreme high altitude flight in the bar-headed goose

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Bar-headed geese migrate over the Himalayas at up to 9000 m elevation, but it is unclear how they sustain the high metabolic rates needed for flight in the severe hypoxia at these altitudes. To better understand the basis for this physiological feat, we compared the flight muscle phenotype of bar-headed geese with that of low altitude birds (barnacle geese, pink-footed geese, greylag geese and mallard ducks). Bar-headed goose muscle had a higher proportion of oxidative fibres. This increased muscle aerobic capacity, because the mitochondrial volume densities of each fibre type were similar between species. However, bar-headed geese had more capillaries per muscle fibre than expected from this increase in aerobic capacity, as well as higher capillary densities and more homogeneous capillary spacing. Their mitochondria were also redistributed towards the subsarcolemma (cell membrane) and adjacent to capillaries. These alterations should improve O₂ diffusion capacity from the blood and reduce intracellular O₂ diffusion distances, respectively. The unique differences in bar-headed geese were much greater than the minor variation between low altitude species and existed without prior exercise or hypoxia exposure, and the correlation of these traits to flight altitude was independent of phylogeny. In contrast, isolated mitochondria had similar respiratory capacities, O₂ kinetics and phosphorylation efficiencies across species. Bar-headed geese have therefore evolved for exercise in hypoxia by enhancing the O₂ supply to flight muscle.

Keywords: oxygen transport cascade; high altitude adaptation; physiological evolution; exercise performance; phylogenetically independent contrasts

1. INTRODUCTION

High altitude environments are particularly challenging to animal life, owing to their characteristically low atmospheric temperatures and O₂ levels. The hypoxia at high altitude can be particularly debilitating for lowland species, such that even basal metabolism is a challenge to sustain. However, numerous species have acquired genetically based physiological adaptations that minimize performance decrements at high altitude (Chappell & Snyder 1984; Beall 2007; Storz & Moriyama 2008). These species provide an exceptional opportunity for studying how complex physiological systems evolve, because the general mechanisms of O₂ transport and utilization are well understood. Because the O₂ transport pathway is composed of a series of cascading physiological steps (breathing, pulmonary diffusion, circulation, tissue diffusion and mitochondrial O₂ utilization) (Weibel 1984), the complex nature of high altitude adaptation is best understood by considering all steps in this pathway.

One of the most celebrated high altitude performers is the bar-headed goose (*Anser indicus*). This species crosses the Himalayas on its biannual migration between southern and central Asia, flying over the highest mountains in the world and reaching altitudes of up to

9000 m (Swan 1970). Incredibly, bar-headed geese sustain the 10–20-fold increase in O₂ consumption rate that is necessary to fuel flapping flight (Ward *et al.* 2002), despite the severe hypoxia at these elevations. The physiological basis for this elevated O₂ transport capacity is not completely understood, but it results in part from evolutionary changes that improve O₂ uptake and circulation during hypoxia (Petschow *et al.* 1977; Jessen *et al.* 1991; Scott & Milsom 2007). Much less is known about the flight muscle of this species, but theoretical modelling suggests that an enhanced capacity for O₂ diffusion from blood into muscle, which can be realized with increased muscle capillarity, should also improve O₂ transport in hypoxia (Scott & Milsom 2006).

Here we demonstrate, using a comparative phylogenetic approach, a striking difference in flight muscle phenotype related to high altitude flight in bar-headed geese. This difference was unique compared with multiple low altitude migratory species and was not due to physiological plasticity or interspecific variation in body mass. The unique flight muscle phenotype in bar-headed geese is therefore inherent and probably serves to improve muscle O₂ transport during flight at extremely high altitudes.

2. MATERIAL AND METHODS

(a) Experimental animals

Histological measurements were made on bar-headed geese (1.9–2.7 kg), barnacle geese (*Branta leucopsis*) (1.6–2.2 kg) and pink-footed geese (*Anser brachyrhynchus*) (2.3–3.5 kg).

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Mitochondrial respiration was performed on bar-headed geese, barnacle geese, greylag geese (*Anser anser*) (3.9–5.1 kg) and mallard ducks (*Anas platyrhynchos*) (1.1–1.6 kg). All birds were young adults of similar age (3–5 years old) that had been bred and raised in captivity at sea level by registered breeders. Measurements were made on both males and females for each species, none of which had ever flown.

All species in this study have a close phylogenetic relationship (figure S1, electronic supplementary material). Barnacle geese, pink-footed geese, greylag geese and mallard ducks all live at low altitudes, and although fine-scale migration patterns are not known for all of these species, they generally follow low-to-moderate altitude migration routes (Cramp & Simmons 1977).

(b) Muscle histology

Birds were terminally anaesthetized with an overdose of intravenously injected sodium pentobarbital, and the pectoralis major muscle was dissected. Samples were taken half way along the length of the sternum, 3–5 cm lateral to the carina, at surface (immediately subcutaneous), intermediate (50% depth) and deep (adjacent to the sternum) muscle depths. Samples were coated in mounting medium and then rapidly frozen in 2-methylbutane (cooled in liquid N₂). Muscle was sectioned (10 µm) transverse to fibre length in a –20°C cryostat. Additional muscle from an intermediate depth was fixed at resting length using 2.5 per cent glutaraldehyde in cacodylate buffer (0.1 M, pH 7.4), then post-fixed in buffered OsO₄ (1%) for 1 h, dehydrated in ethanol and embedded in epoxy resin. Transverse semi-thin sections (0.5 µm) were stained with toluidine blue, and ultrathin sections (approx. 80 nm) were stained with uranyl acetate and lead citrate.

Cryostat sections were stained for succinate dehydrogenase, myosin-ATPase (pre-incubated at pH 4.6) and alkaline phosphatase activities (Deveci *et al.* 2001), and these sections as well as the semi-thin sections (used to identify intramuscular lipid) were imaged using light microscopy. Ultrathin sections were imaged using transmission electron microscopy. Unbiased measurements for determining all histological variables were collected and analysed as described previously (Weibel 1979; Egginton 1990). Sufficient images (five or more) were analysed for each sample to account for heterogeneity, determined by the number of replicates necessary to yield a stable mean value. Mitochondria were classified as subsarcolemmal if they were located between the cell membrane and the outer edges of peripheral myofibrils. Representative alkaline phosphatase-stained images ($n = 4$ for each species, surface muscle depth) were digitized to determine capillary domain area (the area around each capillary whose boundary is equidistant from each adjacent capillary) and an estimate of the heterogeneity of capillary spacing (the coefficient of variation of all domain diameters in an image), both as described previously (Degens *et al.* 1992). A representative image showing capillary domain areas and the more traditional Krogh cylinder areas is shown in figure 1. Average mitochondrial volume density at each muscle depth ($\bar{V}_V(\text{mito}, m)$) was calculated by

$$\bar{V}_V(\text{mito}, m) = V_V(\text{mito}, \text{IIa}) \times A_A(\text{IIa}, m) + V_V(\text{mito}, \text{IIb}) \times A_A(\text{IIb}, m),$$

where $V_V(\text{mito}, \text{IIa})$ and $V_V(\text{mito}, \text{IIb})$ are the mitochondrial volume densities in type IIa and IIb fibres, and $A_A(\text{IIa}, m)$

and $A_A(\text{IIb}, m)$ are the areal densities of the type IIa and IIb fibres.

(c) Respiration of muscle mitochondria

A mixed population of subsarcolemmal and intermyofibrillar mitochondria were isolated from various muscle depths. Briefly, birds were terminally anaesthetized, and a large sample (approx. 6 g) of muscle was removed, immediately transferred to 25 ml ice-cold isolation buffer (in mM: 100 sucrose, 50 Tris base, 5 MgCl₂, 5 EGTA, 100 KCl, 1 ATP; pH 7.4) and minced. Muscle was digested for 5 min with nagarse (1 mg g⁻¹ muscle) and homogenized with five passes of a Potter–Elvehjem homogenizer (100 r.p.m.). Homogenate was centrifuged at 1000g for 10 min at 4°C. The supernatant was filtered through cheesecloth and centrifuged at 8700g for 10 min at 4°C (the same for all further centrifugation). The pellet was resuspended in 20 ml isolation buffer and centrifuged, resuspended in 10 ml storage buffer (in mM: 0.5 EGTA, 3 MgCl₂, 60 K-methanesulphonate, 20 taurine, 10 KH₂PO₄, 20 HEPES, 110 sucrose, 1 MgCl₂, 0.02 vitamin E succinate, 2 pyruvate, 2 malate; pH 7.1), centrifuged and resuspended in 3 ml storage buffer. Mitochondria were stored on ice until experimentation.

Respiration of mitochondria (0.04 mg mitochondrial protein) was measured by high-resolution respirometry (Oxygraph-2k, Oroboros) in 2 ml of respiration buffer (in mM: 0.5 EGTA, 3 MgCl₂, 60 K-MES, 20 taurine, 10 KH₂PO₄, 20 HEPES, 110 sucrose, 1 MgCl₂, 1 g l⁻¹ fatty acid-free bovine serum albumin; pH 7.1) at avian body temperature (41°C). Two separate experiments were performed using two different combinations of substrates. In experiment 1, state 2 was first stimulated with malate (2 mM) and pyruvate (5 mM). *P/O* ratios were determined twice by the conventional method (Gnaiger *et al.* 2000) by adding 125 µM of ADP. After state 4 respiration was reached, state 3 was induced with maximal ADP (0.7–1.0 mM), and mitochondria were allowed to deplete all O₂. After a period of anoxia (5 min), the O₂ tension was raised, the remaining ADP was consumed and all O₂ was consumed in state 4. After anoxia, the O₂ tension was raised, cytochrome oxidase was maximally stimulated with ADP (1.25 mM), TMPD (*N,N,N',N'*-tetramethyl-*p*-phenylenediamine; 0.5 mM) and ascorbate (0.5 mM) and all O₂ was consumed. After anoxia, the O₂ tension was raised and mitochondria were uncoupled with FCCP (carbonylcyanide-*p*-trifluoromethoxyphenylhydrazone; 0.5 µM). Experiment 2 was the same as experiment 1, except that succinate (10 mM) was also present to spark state 2. The O₂ tension that reduced mitochondrial respiration to 50 per cent of the normoxic rate (P_{50}) was determined for states 3 and 4, and for TMPD-stimulated respiration, as previously described (Gnaiger 2001). Respiration rates were corrected for background O₂ flux and are expressed relative to mitochondrial protein concentration.

(d) Statistical analyses

Data are generally reported as means $\pm$ s.e. One- or two-factor ANOVA and Student–Newman–Keuls *post hoc* tests were used, as appropriate, and abnormally distributed data were log transformed before testing. Ordinary least squares (OLS) and reduced major axis (RMA) linear regressions were used for correlations. RMA regressions were calculated with the program RMA (Bohonak & van

Figure 1. Histochemical staining of goose pectoralis muscle, showing that only fast oxidative (type IIa) fibres (arrow) and fast glycolytic (type IIb) fibres (asterisk) are present. (a) Succinate dehydrogenase (SDH), a mitochondrial enzyme that depicts oxidative capacity. (b) Myosin-ATPase (pH 4.6) to identify different muscle fibre types. (c) Alkaline phosphatase to identify capillaries. Inset is a representative digitization of capillaries and their domain areas (blue), an analysed region of interest (green) and the Krogh cylinder areas for capillaries within that region (magenta) (see §2). Scale bar, 100 μm .

der Linde 2004). All other traditional statistical tests were performed using Sigmapstat (Systat).

We also performed phylogenetically independent contrast analysis (Midford *et al.* 2005; Maddison & Maddison 2009) for traits that were uniquely different in bar-headed geese.

We used our previously published phylogenetic tree with branch lengths (Lee *et al.* 2008) pruned to include only the species in question (figure S1, electronic supplementary material). Low and high altitude migration strategies were coded as 0 or 1 dummy variable (Garland *et al.* 1992), respectively. We tested for significant positive relationships between the standardized independent contrasts of muscle traits and flight altitude by determining the one-tailed p -values for correlations computed through the origin (d.f. = 1 for all). Setting branch lengths to 1 yielded very similar results and is therefore not shown. A significance level of $p < 0.05$ was used throughout.

3. RESULTS AND DISCUSSION

(a) Flight muscle phenotype in geese

The flight muscle of geese contained fast oxidative (type IIa) and fast glycolytic (type IIb) fibre types, which exhibit stereotypical differences in succinate dehydrogenase (a mitochondrial enzyme) and myosin-ATPase (an index of contractility) activities (figure 1). Consistent with what is known for other bird species (Rosser *et al.* 1987; Torrella *et al.* 1998), type IIa fibres were most abundant across the muscle, and slow oxidative (type I) fibres were not observed.

There was a striking difference in the muscle fibre composition of bar-headed geese compared with the low altitude birds (figure 2 and table 1; figures S2 and S3, electronic supplementary material). The flight muscle of barnacle and pink-footed geese had a significant proportion of fast glycolytic fibres near the muscle surface, but as muscle depth increased so too did the proportion of fast oxidative fibres; this appears to be a general characteristic of birds (Mathieu-Costello *et al.* 1998a). In contrast, the flight muscle of bar-headed geese had a much higher proportion of fast oxidative fibres. This was apparent for average values across the whole muscle (bar-headed geese, $82.5 \pm 0.9\%$; barnacle geese, $76.8 \pm 1.0\%$; pink-footed geese, $71.2 \pm 2.3\%$; d.f. = 21) and was exaggerated near the muscle surface, where the potential for increasing oxidative fibre density was greatest.

Consistent with the differences in fibre composition, the flight muscle of bar-headed geese had more capillaries than that of low altitude geese (figure 3; electronic supplementary material, figure S4). Both the number of capillaries per fibre ($C:F$) and the capillary density (CD) increased with muscle depth in barnacle and pink-footed geese. $C:F$ was much higher in bar-headed geese at surface and intermediate muscle depths, as well as for the global average (bar-headed geese, 2.13 ± 0.09 ; barnacle geese, 1.72 ± 0.08 ; pink-footed geese, 1.69 ± 0.07 ; d.f. = 21), such that it was as high in the superficial regions as in the deep regions. CD was also higher in bar-headed geese than in both low altitude species near the muscle surface, and the overall average was higher than in pink-footed geese (bar-headed geese, $2287 \pm 112 \text{ mm}^{-2}$; barnacle geese, $2270 \pm 118 \text{ mm}^{-2}$; pink-footed geese, $1802 \pm 107 \text{ mm}^{-2}$; d.f. = 21). The less pronounced interspecific differences in CD than in $C:F$ probably resulted from a confounding effect of allometry on the muscle fibre size of barnacle geese (table 1; figure S5, electronic supplementary material). Nevertheless, consistent with the differences in CD near the muscle surface, the volume of muscle supplied by

Figure 2. Muscle fibre composition is altered in the pectoralis muscle of bar-headed geese (grey bar) ($n = 6$) compared with barnacle geese (unfilled bar) ($n = 8$) and pink-footed geese (black bar) ($n = 8$). High SDH activity identified oxidative fibres, and high acid-stable myosin-ATPase activity identified type IIa fibres. * and † represent significant differences from both low altitude species or just pink-footed geese, respectively (two-way ANOVA, d.f. = 65).

each capillary (as indicated by the capillary domain areas) was smaller in bar-headed geese than in both low altitude species (table 1). Muscle capillaries also appeared to be more homogeneously spaced in bar-headed geese, because the coefficient of variation of capillary spacing in this species was significantly less than that in barnacle geese and lower on average than that in pink-footed geese (table 1).

We also performed phylogenetically independent contrast analysis on muscle traits that were uniquely different in bar-headed geese, by testing for positive relationships between the standardized contrasts of these traits and flight altitude (figure S6; see §2). There were significant positive relationships between flight altitude and oxidative fibre areal density (Pearson product-moment correlation coefficient, $r = 0.996$), myosin-ATPase type IIa areal density ($r = 0.999$) and CD ($r = 0.994$), all at the muscle surface, as well as $C:F$ at the surface ($r = 0.998$), intermediate ($r = 0.989$) and deep ($r = 0.999$) muscle depths. Therefore, the associations between the unique characteristics of bar-headed geese and their high altitude migration strategy are independent of phylogeny.

Because differences in fibre composition *per se* will influence capillarity (owing to concomitant changes in O_2 demands), we next sought to determine whether the increased proportion of oxidative fibres could account for the increased capillarity in bar-headed goose muscle. To do so, we first assessed the mitochondrial abundance in oxidative and glycolytic fibres in all species (figure S7, electronic supplementary material). There were no statistically significant differences between species in the mitochondrial volume densities within oxidative or glycolytic fibres, or in the densities of intramuscular or intracellular lipid droplets (table 1). Mitochondrial cristae surface densities were high (approx. $60 \mu m^2 \mu m^{-3}$), similar to those in the highly aerobic flight muscle of hummingbirds (Suarez *et al.* 1991), but were also not different between species. With these data, we then calculated the average mitochondrial volume density at each muscle depth for each species. $C:F$ was more strongly related to average mitochondrial volume density (figure 3) than CD (figure S8, electronic supplementary material) when only low altitude species were considered and was thus a better index for comparing capillarity between species. In doing so, we found that bar-headed geese had more capillaries per muscle fibre for a given mitochondrial volume density at surface and intermediate muscle depths. Therefore, capillarity and O_2 diffusion capacity are higher in bar-headed goose muscle than can be accounted for by the increased proportion of oxidative fibres.

This result has clear benefits for sustaining muscle O_2 flux when capillary O_2 tensions fall during hypoxia. Although $C:F$ is higher in birds living at high altitude (León-Velarde *et al.* 1993), in some cases, this has been entirely attributed to concurrent increases in mitochondrial abundance (Mathieu-Costello *et al.* 1998b). Furthermore, exercise and hypoxia would have been potential stimulants of angiogenesis in these studies, in contrast to the inherently higher capillarity that exists in bar-headed geese before ever flying or experiencing high altitude. Therefore, despite some disagreement about its role during high altitude acclimatization (Mathieu-Costello 2001), increases in muscle O_2 diffusion capacity have evolved in some high altitude species and probably improve aerobic performance in hypoxia.

Although mitochondrial abundance within each fibre type is similar between species, the proportion of all mitochondria that are subsarcolemmal (rather than intermyofibrillar) was much higher in bar-headed geese (figure 4). Furthermore, the positive relationship between subsarcolemmal mitochondria proportion and flight altitude persisted after correcting for phylogeny ($r = 0.999$ for correlation shown in figure S6, electronic supplementary material). This redistribution of mitochondria towards the cell membrane and closer to capillaries should reduce intracellular diffusion distances. Its importance for improving O_2 transport is emphasized by previous human studies showing that increases in aerobic performance after exercise training are associated with a preferential proliferation of subsarcolemmal mitochondria (Hoppeler *et al.* 1985). In fact, the proportion of subsarcolemmal mitochondria in bar-headed goose muscle is even higher than in the highly aerobic flight muscle of hummingbirds (Suarez *et al.* 1991). However, this mitochondrial redistribution could hinder the

Table 1. Histological measurements from the flight muscle of geese.

muscle trait	muscle location	bar-headed geese	barnacle geese	pink-footed geese
oxidative fibre numerical density (%) ^a	surface	83.5 ± 1.5 ^b	76.0 ± 1.5	76.4 ± 2.1
	intermediate	92.5 ± 0.7 ^c	89.7 ± 1.0	84.2 ± 2.5
	deep	95.1 ± 0.3	93.9 ± 0.5	92.2 ± 1.5
myosin-ATPase IIa numerical density (%) ^a	surface	81.9 ± 1.3 ^b	75.0 ± 2.7	76.1 ± 2.8
	intermediate	91.3 ± 0.7 ^c	91.1 ± 0.8	84.3 ± 1.8
	deep	96.2 ± 0.4	94.6 ± 0.4	91.9 ± 0.9
IIa fibre transverse area (µm ²) ^d		852 ± 78	683 ± 43	798 ± 45
IIb fibre transverse area (µm ²) ^d		1797 ± 202	1504 ± 119	1892 ± 157
capillary domain area (µm ²) ^e		536 ± 25 ^b	648 ± 21	872 ± 28
coefficient of variation for capillary spacing (%) ^e		15.1 ± 0.4 ^f	20.3 ± 1.8	17.8 ± 1.7
IIa (fast oxidative) fibre mitochondrial volume density ^g		0.25 ± 0.01	0.27 ± 0.01	0.25 ± 0.01
IIb (fast glycolytic) fibre mitochondrial volume density ^g		0.053 ± 0.003	0.056 ± 0.005	0.054 ± 0.006
mitochondrial cristae surface density (µm ² µm ⁻³) ^g		59.8 ± 0.9	61.5 ± 0.8	62.4 ± 1.3
IIa fibre intracellular lipid volume densities ^g		0.020 ± 0.005 ^c	0.017 ± 0.003 ^c	0.051 ± 0.01
IIb fibre intracellular lipid volume densities ^g		0.007 ± 0.001	0.008 ± 0.002	0.006 ± 0.003
intramuscular lipid volume densities ^g		0.024 ± 0.007	0.020 ± 0.003	0.019 ± 0.004

^a*n* and d.f. as in figure 2.^bSignificant difference from both low altitude species.^cSignificant difference from just pink-footed geese.^d*n* as in figure 2, d.f. = 21.^e*n* = 4 for each species, d.f. = 11.^fSignificant difference from just barnacle geese.^g*n* and d.f. as in figure 4.

intracellular movement of ATP and other metabolites (Kinsey *et al.* 2007), which suggests that bar-headed goose flight muscle may need an effective system for shuttling ATP equivalents, such as the creatine kinase shuttle (Andrienko *et al.* 2003).

Although the mechanisms accounting for the altered muscle phenotype in bar-headed geese are unclear, they may involve inherent differences in muscle development (Bassel-Duby & Olson 2006; Biressi *et al.* 2007). Activity-dependent regulators of muscle phenotype (e.g. neural stimulation) did not cause the differences in this study because no species had been allowed to fly. The differences probably instead reflect evolutionary changes in the gene networks regulating skeletal muscle. Flight muscle aerobic capacity develops rapidly after birth (Bishop *et al.* 1995), and it is probable that bar-headed geese acquire their altered phenotype in the short time before their first migration.

(b) Respiration of muscle mitochondria in geese

The maximum capacities for respiration of mitochondria isolated from the flight muscle were extremely similar between bar-headed geese and low altitude species (figure 5). This was assessed for the entire electron transport chain (state 3, maximal ADP stimulation), for cytochrome oxidase (treatment with TMPD, an exogenous electron donor) and for ATP synthase (treatment with FCCP, a mitochondrial uncoupler). These results suggest that the alteration in fibre composition (figure 2), rather than any change in the respiration or density of mitochondria within muscle cells, is the primary factor increasing aerobic capacity in bar-headed goose muscle. Bar-headed geese were also no more effective at making ATP, as indicated by an equivalent

phosphorylation efficiency (indicated by the *P/O* ratio) to that of other species (figure 5).

The effects of low oxygen on mitochondrial respiration were largely similar between species (figure 6). This was particularly true at high respiration rates (state 3 or cytochrome oxidase stimulation), as would occur during flight exercise, although bar-headed geese had the highest mitochondrial *P*₅₀ at low respiration rates (state 4). Overall, the state-dependent differences in O₂ kinetics resulted in a strong positive relationship between *P*₅₀ and respiration rate across all species (figure S9, electronic supplementary material). The *P*₅₀ values we observed were slightly higher than those for mammalian mitochondria (Gnaiger 2001), potentially because we used a higher temperature (avian body temperature of 41°C).

There is a well-known hyperbolic relationship between O₂ tension and mitochondrial respiration rate (Gnaiger 2001) (shown in figure S9, electronic supplementary material). Mild O₂ limitation (e.g. inhibition by approx. 5–10%) will therefore occur at much higher O₂ tensions than *P*₅₀ (i.e. 50% inhibition), which was approximately 1 Torr in state 3. However, muscle intracellular O₂ tensions (PiO₂) during intense exercise in normoxia can be well below 5 Torr in mammals (Gayeski & Honig 1988). This suggests that bird muscle mitochondria may be O₂ limited during flight, particularly during environmental hypoxia or vigorous exercise. This emphasizes the importance of the enhanced mitochondrial O₂ supply in bar-headed geese for sustaining respiration during high altitude flight.

The conservation of mitochondrial *P*₅₀ across species also clarifies why bar-headed geese could benefit from having more oxidative fibres. In the absence of any change in O₂ kinetics, improvements in muscle aerobic capacity can increase the total mitochondrial O₂ flux of

Figure 3. Capillarity is enhanced in the pectoralis muscle of bar-headed geese. (a) Capillary density and capillary-to-muscle-fibre ratio at different muscle depths (symbols and statistical features as in figure 2). (b) Within low altitude species, capillary-to-fibre ratios were strongly predicted by average mitochondrial volume density across the three muscle depths, for both OLS (solid line; $y = (8.03 \pm 1.02)x + (0.068 \pm 0.213)$, $R^2 = 0.939$) and RMA (black dashed line; $y = (7.29 \pm 1.48)x + (0.198 \pm 0.308)$, $R^2 = 0.863$) regressions (d.f. = 5). However, bar-headed geese were above the OLS 95 per cent prediction intervals (grey dashed lines) at surface and intermediate depths. Triangle, bar-headed goose; open circle, barnacle goose; filled square, pink-footed goose.

Figure 4. Mitochondria are redistributed towards the cell membrane in the oxidative fibres of bar-headed goose muscle. (a) The proportion of mitochondria that were subsarcolemmal was higher in bar-headed geese ($n = 5$; *) than in both low altitude species ($n = 4$ each; one-way ANOVA, d.f. = 12). Grey bar, bar-headed goose; unfilled bar, barnacle goose; black bar, pink-footed goose. (b,c) Representative transmission electron micrographs of oxidative fibres from (b) bar-headed geese and (c) barnacle geese. Scale bar, 2 μm . Arrow, subsarcolemmal mitochondria; arrowhead, intermyofibrillar mitochondrion.

the entire muscle at a reduced PiO_2 (Hochachka 1985). In this way, having more oxidative fibres may counterbalance the inhibition of respiration by intracellular hypoxia in each individual fibre. This would make bar-headed geese less reliant on anaerobic metabolism for sustaining ATP turnover during flight in hypoxia, thus improving fatigue resistance. Their muscle may also be less inclined towards carbohydrate oxidation, as normally occurs at higher exercise intensities (McClelland *et al.* 1998), which is not as sustainable a fuel source for long distance migration as fats.

(c) Evolution of O_2 transport for flight at high altitude

Our results are consistent with the idea that muscle phenotype evolved in bar-headed geese as an important adaptation for flying at extremely high altitudes. Increasing the capacity for O_2 diffusion from the blood and reducing the intracellular diffusion distance should both improve O_2 transport and thus exercise performance in hypoxia. The unique phenotype of bar-headed geese is clearly not a product of phylogenetic history: differences between low altitude species were generally small, particularly when compared with the large differences between these species and bar-headed geese; furthermore, the associations between these muscle traits and high altitude flight were independent of phylogeny. The

Figure 5. Mitochondria isolated from the flight muscle of bar-headed geese ($n = 6$, dark grey bar), greylag geese ($n = 4$, black bar), barnacle geese ($n = 3$, unfilled bar) and mallard ducks ($n = 9$, light grey bar) had similar respiration rates and phosphorylation efficiencies. Two different combinations of malate (mal), pyruvate (pyr) and succinate (succ) were used. (a) Significant increases in respiration occurred in all species as mitochondria were transitioned from state 2 (no ADP or ATP) to state 4 (all ADP converted to ATP), state 3 (maximal ADP stimulation) and TMPD (electron donor that maximally stimulates cytochrome oxidase) treatment, but FCCP (mitochondrial uncoupler) did not increase respiration any further (two-way ANOVA within each substrate combination, d.f. = 109; substrate combinations were not compared). (b) P/O ratios (ADP/oxygen atom consumed) were significantly lower when succinate was present (two-way ANOVA, d.f. = 43).

Figure 6. Oxygen kinetics of mitochondria isolated from the flight muscle was similar in bar-headed geese to other bird species (n and statistical features as in figure 5). Mitochondrial P_{50} is the O₂ tension that causes 50 per cent inhibition of normoxic respiration and was determined for state 3 (maximal ADP stimulation), state 4 (no ADP) and TMPD (electron donor that maximally stimulates cytochrome oxidase) treatment. Two different combinations of malate (mal), pyruvate (pyr) and succinate (succ) were used (not compared statistically). P_{50} was significantly different between respiration states (two-way ANOVA within each substrate combination, d.f. = 65). 1 Torr = 0.133 kPa. †, ** and †† represent significant differences from mallard ducks, greylag geese and mallard ducks, or all other species, respectively.

unique features of bar-headed geese do not result from phenotypic plasticity either, because all species were born and raised in equivalent conditions at sea level. Differences in body size had small effects on muscle fibre size, but because bar-headed geese were

intermediate in mass between the two low altitude species, allometric variation cannot explain their unique muscle phenotype. It is therefore likely that the changes in muscle phenotype were uniquely derived in bar-headed geese and enhanced flight performance at high altitude. However, evolutionary forces other than high altitude adaptation could account for this change, such as genetic correlations with other phenotypes or selection for performance traits other than high altitude flight (Lauder *et al.* 1993). A stronger case for adaptation could be made if other high altitude waterfowl species had similar specializations for improving muscle O₂ transport.

The differences in muscle phenotype and O₂ diffusion capacity are part of a suite of evolutionary changes in the O₂ transport pathway of bar-headed geese that improve O₂ flux in hypoxia. One of these alterations, an inherently higher haemoglobin O₂ affinity (Petschow *et al.* 1977), is known to be caused largely by a single amino acid substitution (Jessen *et al.* 1991; Zhang *et al.* 1996). This improves O₂ transport in hypoxia by enhancing pulmonary O₂ uptake (Scott & Milsom 2007) and assuring better O₂ delivery throughout the body (Faraci *et al.* 1984). Pulmonary O₂ uptake is also improved in bar-headed geese owing to an enhanced hypoxic ventilatory response and a more effective breathing pattern (Scott & Milsom 2007; Scott *et al.* 2008). Our present findings therefore imply that improvements in pulmonary loading and delivery of O₂ occur in conjunction with enhanced O₂ extraction from the blood by the flight muscle in bar-headed geese.

In addition to helping explain how this unique species can fly in severe hypoxia at high altitude, our present findings shed insight into how respiratory systems evolve. Early theories for how the O₂ transport pathway evolves suggested that the capacity of every step in the pathway must increase equally to improve overall capacity (i.e. symmorphosis) (Weibel *et al.* 1991). More recent theories of respiratory pathway flux argue that overall control arises from the summed influence of each step in the pathway and that different steps have unequal contributions to control (Hochachka & Burelle 2004). This implies by extension that changes in the overall capacity for O₂ transport during evolution can involve changes of varying magnitudes at different steps. Our work with bar-headed geese supports the idea that respiratory system evolution occurs through changes at multiple interacting steps in the O₂ transport pathway. In the light of previous work in this species, our present data suggest that the physiological traits having the greatest control over O₂ transport in hypoxia (Scott & Milsom 2006) are also the most likely to evolve and improve exercise performance at high altitudes.

4. CONCLUSIONS

Our present findings suggest that O₂ diffusion capacity in the flight muscle is enhanced in bar-headed geese, owing to both an increase in the number of capillaries surrounding each muscle fibre and a redistribution of mitochondria towards the cell membrane within the fibres, and thus closer to capillaries. Muscle aerobic capacity is also improved by an increased proportion of oxidative fibres, but properties of mitochondrial O₂ demand are otherwise conserved between species. The flight muscle of bar-headed geese has therefore evolved

for exercise in severe hypoxia by enhancing muscle O₂ supply, which may be especially important for this species' incredible ability to fly high.

All animal care and experimentation was conducted according to UBC animal care protocol no. A04-1013.

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