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RESEARCH ARTICLE

Consistent changes in muscle phenotype and mitochondrial abundance underlie dive performance across multiple lineages of diving ducks

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ABSTRACT

Diving animals must sustain high muscle activity with finite oxygen (O_2) to forage underwater. Studies have shown that some diving mammals exhibit changes in the metabolic phenotype of locomotory muscles compared with non-divers, but the pervasiveness of such changes across diving animals is unclear, particularly among diving birds. Here, we examined whether changes in muscle phenotype and mitochondrial abundance are associated with dive capacity across 17 species of ducks from three distinct evolutionary clades (tribes) in the subfamily Anatinae: the longest diving sea ducks, the mid-tier diving pochards and the non-diving dabblers. In the gastrocnemius (the primary swimming and diving muscle), mitochondrial volume density in both oxidative and glycolytic fiber types was 70% and 30% higher in sea ducks compared with dabblers, respectively. These differences were associated with preferential proliferation of the subsarcolemmal subfraction, the mitochondria adjacent to the cell membrane and nearest to capillaries, relative to the intermyofibrillar subfraction. Capillary density and capillary-to-fiber ratio were positively correlated with mitochondrial volume density, with no variation in the density of oxidative fiber types across tribes. In the pectoralis, sea ducks had greater abundance of oxidative fiber types than dabblers, whereas pochards were intermediate between the two. These data suggest that skeletal muscles of sea ducks have a heightened capacity for aerobic metabolism and an enhanced ability to utilize O_2 stores in the blood and muscle while diving.

KEY WORDS: Mitochondria, Locomotion, Exercise performance, Hypoxia, Muscle ultrastructure

INTRODUCTION

One of the most pressing issues facing air-breathing vertebrates that forage underwater is the lack of access to breathable oxygen (O_2) while diving. In non-diving vertebrates, the cardiorespiratory system provides a continuous supply of O_2 to meet the demands of locomotory muscles (Hepple, 2000; Hochachka et al., 1983). Diving vertebrates, in contrast, must sustain aerobic metabolism using the limited O_2 stores in the lungs, blood and tissue. Studies on free-diving vertebrates have shown that most dives stay within the aerobic dive limit, indicating that aerobic metabolism is typically sustained for the duration of an underwater foraging bout (Kooyman et al., 1980; Ponganis et al., 1997b). However, O_2 levels decrease at later stages of a dive, whereas muscle activity, and thus metabolism, may not. These factors have likely driven the evolution of a range of strategies to match O_2 supply and demand in diving vertebrates.

Balancing O_2 supply and demand while diving can occur through cardiovascular adjustments, metabolic regulation and increases in overall O_2 storage capacity (Kooyman and Ponganis, 1998; Lenfant et al., 1970; Meir and Ponganis, 2009). Because underwater locomotion is metabolically demanding, conserving O_2 via reductions in metabolic rate is not seen in diving vertebrates. In fact, the activity of mitochondrial enzymes is often increased in the locomotory muscles of diving animals, most notably citrate synthase (Butler and Turner, 1988; Kanatous et al., 1999; Schell et al., 2023). Studies of some diving taxa have shown that these increases in mitochondrial enzyme activities are associated with increases in mitochondrial volume density (Kanatous et al., 1999; Ponganis et al., 1997a), which may serve to increase the capacity for oxidative phosphorylation supported by blood- and muscle-O_2 stores.

Within skeletal muscle, there are two distinct mitochondrial fractions, which are characterized based on their location in the cell and biochemical properties. The subsarcolemmal mitochondria (also referred to as paravascular mitochondria) are located directly adjacent to the cell membrane, and the intermyofibrillar mitochondria are located deeper in muscle fibers among the myofibrils (Palmer et al., 1977). Subsarcolemmal mitochondria have been found to have lower enzyme activities (e.g. citrate synthase, ATP synthase) and a greater relative capacity for oxidizing lipids over carbohydrates compared with intermyofibrillar mitochondria (Dawson and Scott, 2022; Palmer et al., 1977). Owing to their location adjacent to the cell membrane, increasing the abundance of subsarcolemmal mitochondria may be particularly advantageous for utilizing blood-O_2 stores within capillaries. However, examinations of intracellular mitochondria distribution have so far been limited to relatively few diving species (Kanatous et al., 1999, 2008; Kielhorn et al., 2013; Watson et al., 2007). Thus, it remains unclear whether consistent changes in mitochondrial abundance and distribution in muscle have occurred across divers. Very few previous studies on this subject have focused on diving birds (Ponganis et al., 1997a; Turner and Butler, 1988).

We might also expect to see increases in capillarity in the locomotory muscles of diving vertebrates to facilitate the delivery and use of O_2 stores in the blood. The opposite trend is seen in diving mammals, with capillarity reduced in swimming muscles, perhaps due to the reduction in blood flow from peripheral vasoconstriction and decreased heart rate while diving. This decrease in O_2 delivered

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by the blood could be offset by a strong reliance on myoglobin-bound O_2 stores (Davis, 2014; Kanatous et al., 2001). The few ducks that have been studied, however, exhibit a much more pronounced elevation in heart rate above resting levels and muscle perfusion upon submersion (Bevan and Butler, 1992; Stephenson et al., 1986). Thus, we might expect to see increases in capillarity to support aerobic muscle activity, although capillary density in the gastrocnemius in the diving tufted duck (*Aythya fuligula*) was not significantly different from that of the non-diving mallard (*Anas platyrhynchos*) (Torrella et al., 1996; Turner and Butler, 1988).

Variation in muscle traits such as mitochondrial volume density and capillarity could result from phenotypic plasticity or from natural selection and evolutionary diversification. For example, mitochondrial volume density and muscle capillarity can increase as a result of exercise training (Butler and Turner, 1988; Hoppeler, 1986), so the muscle phenotype of divers is likely influenced by their level of activity, which can also vary seasonally. Such changes in mitochondrial volume density can occur rapidly. For example, barnacle geese experience rapid increases in mitochondrial density in the pectoralis (as reflected by citrate synthase activity) over the few weeks leading up to migration (Bishop et al., 1995). Furthermore, little is known about differences between the sexes in birds. In addition to seasonal shifts in individuals, evolved changes in mitochondrial volume density and intracellular distribution can arise from hypoxia adaptation, such as has occurred in some high-altitude taxa (Mahalingam et al., 2017; Scott et al., 2009). Studies of animals in their native environment are key first steps towards understanding the combined influence of plasticity and adaptation on the muscle phenotype of diving animals.

Here, we used the taxonomically diverse waterfowl subfamily Anatinae (Livezey, 1997) as a novel study system to investigate diving adaptations across several avian lineages. The species in this subfamily encompass a wide range of diving abilities, from the non-diving dabbling ducks (Tribe Anatini) to the common eider (*Somateria mollissima*), with a recorded maximum dive duration of 80 s (Guillemette et al., 2004), or the long-tailed duck (*Clangula hyemalis*), which can dive to 55 m depth or more (Schorger, 1947). Within the Anatinae, diving has evolved separately in multiple lineages, including once in the sea ducks (Tribe Mergini) and once in the pochards (Tribe Aythyini) (McCracken et al., 1999). Phylogenetic analysis suggests that the sea ducks, not pochards, represent the more basal lineage and thus the earlier origin of these two diving clades, which are not sister taxa (Gonzalez et al., 2009; Sun et al., 2017).

Inclusion of members of all three clades in the present study enabled us to investigate whether the unique pressures of breath-hold diving have led to convergent changes in muscle phenotype. In the 17 species studied, a range of dive capabilities were represented: the longest and deepest diving sea ducks (nine species), the mid-tier diving pochards (three species) and the non-diving dabblers (five species) (Fig. 1). We used these three tribes to investigate how the evolution of diving has restructured the phenotype of skeletal muscles. We compared the abundance and distribution of mitochondria, fiber-type composition and capillarity in the gastrocnemius, as all the diving species here are known to be primarily leg-propelled divers (Lovvorn, 1991). We also complemented these detailed analyses with a comparison of fiber-type composition in the pectoralis major, the primary flight muscle, owing to the importance of flight as a primary mode of locomotion for these species as well, as well as secondary locomotion for species that use their wings during dives. All 17 species studied share a close evolutionary history, so we utilized a phylogenetic framework for all statistical analyses to account for the non-independence of individual observations (Felsenstein, 1985; Garland et al., 1993).

MATERIALS AND METHODS

Specimen collection

Tissue samples were taken from five to eight individuals from each of the 17 species studied. All species except harlequin ducks, surf scoters and common eiders were collected in Alaska between 2019 and 2021 from ponds and rivers surrounding Fairbanks, the Dalton and Denali Highways, and Kodiak. Harlequin ducks and surf scoters were collected in Prince William Sound in March 2020, and common eiders were collected in Nome during May 2022. We standardized the months of sample collection to the spring period between March to May to include only adults and to minimize the potential influence of phenotypic variation owing to seasonal changes in activity (McKechnie, 2008). Upon collection, two tissue samples from the gastrocnemius and one from the pectoralis were immediately dissected and prepared for either transmission electron microscopy (TEM) or succinate dehydrogenase (SDH) staining. Previous work has shown that the fiber-type composition of both the pectoralis and gastrocnemius varies with depth in the tissue, and that intermediate depths are most representative of the average composition of the entire muscle (Torrella et al., 1996, 1998). Therefore, we sampled from the same intermediate depth halfway between the surface of the muscle and the bone for all analyses, as done previously for enzyme analyses (Dawson et al., 2020; Schell et al., 2023).

All specimen collection and import/export was conducted with permits from the US Fish and Wildlife Service (MB33283C, MB812229, MB836720), Alaska Department of Fish and Game (19-091, 19-153, 20-033, 20-091, 21-103), Environment and

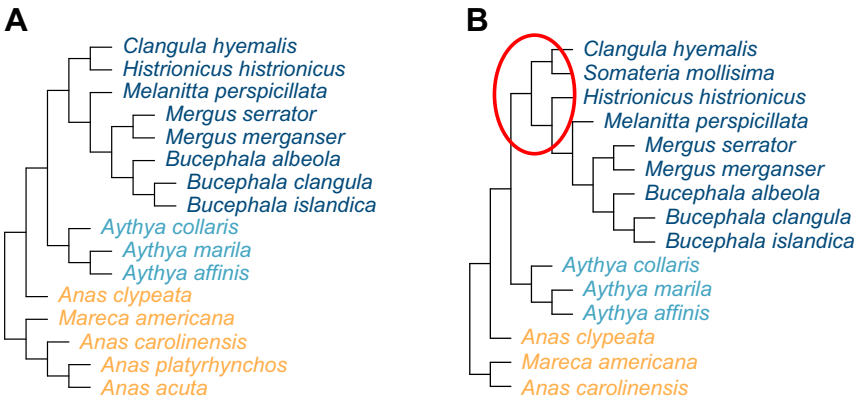

Fig. 1. Phylogeny reconstructions for the oxidative fiber numerical density and mitochondrial abundance analyses. (A) Species tree for oxidative fiber numerical density, with the topology originally published in Schell et al. (2023) using 10,731 overlapping autosomal ddRADseq loci. (B) Our original topology for mitochondrial abundance, updated to include the common eider (*Somateria mollissima*) based on the topology of Lavretsky et al. (2021) and to remove the mallard (*Anas platyrhynchos*) and northern pintail (*Anas acuta*). Branch lengths have been set to 1 for uniformity in analyses in both trees. The red circle indicates where nodes differ from the original topology. Tribes are indicated by color: sea ducks, dark blue; pochards, teal; and dabblers, gold.

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Climate Change Canada (SC-OR-2021-POS 369, SC-OR-2022-POS 369), and Institutional Animal Care and Use Committee (IACUC) from the University of Miami (17-107, 20-090).

TEM imaging and analysis

TEM was used to visualize and quantify mitochondrial abundance and distribution as well as capillarity. In the field, samples dissected from the gastrocnemius were fixed at resting length (no tension or compression applied) with 2% glutaraldehyde in a 0.1 mol l^{-1} sodium cacodylate buffer (pH 7.4), and stored on ice until transfer to a refrigerator. Fixed muscle tissue was then washed in buffer, post-fixed in 1% osmium tetroxide in a 0.1 mol l^{-1} sodium cacodylate buffer, dehydrated in ethanol, and embedded in Spurr's resin for overnight polymerization at 60°C. Ultrathin sections (~80 nm) were cut using a Leica UCT ultramicrotome (Leica, Wetzlar, Germany) and stained with uranyl acetate and lead citrate. All TEM images were analyzed using FIJI v. 2.1.0 (Schindelin et al., 2012).

Transmission electron micrographs of entire muscle fibers were taken for 10 oxidative and 10 glycolytic fibers per individual using a JEOL JEM 1200 EX TEMSCAN transmission electron microscope (JEOL, Peabody, MA, USA) at a magnification of 2000×. A previous study showed that glycolytic fibers are 1.4–2.0× larger than oxidative fibers in the mallard gastrocnemius (Torella et al., 1999), so we distinguished fiber types based on size. *Post hoc* assessment of mitochondrial volume densities in each of these two fiber size classes (as determined below) confirmed that the smaller group of fibers within each individual bird always had higher overall densities of mitochondria and higher densities of subsarcolemmal mitochondria than the larger group of fibers. We determined the volume density of the subsarcolemmal and intermyofibrillar subfractions of mitochondria for each fiber using a 3 μm^2 area grid and the point count method of Weibel (1979). Subsarcolemmal mitochondria were determined visually as those directly adjacent to the cell membrane outside the myofibrils, whereas intermyofibrillar mitochondria were those located between the contractile filaments. A minimum of five images per individual was used to obtain a stable mean for each parameter. Average mitochondrial volume density for the gastrocnemius ($V_{\mathrm{Vmito,m}}$) was calculated according to Scott et al. (2009):

$$V_{\mathrm{Vmito,m}} = V_{\mathrm{Vmito,ox}} \times A_{\mathrm{Aox,m}} + V_{\mathrm{Vmito,gly}} \times A_{\mathrm{A\,gly,m}}, \quad (1)$$

where $V_{\mathrm{Vmito,ox}}$ and $V_{\mathrm{Vmito,gly}}$ are the volume densities of mitochondria in oxidative and glycolytic fibers, and $A_{\mathrm{Aox,m}}$ and $A_{\mathrm{Agly,m}}$ are the areal densities of each fiber type in the gastrocnemius (see below). Transverse area for each fiber type was determined as image area (μm^2) multiplied by the number of grid points overlaying a fiber, divided by the total number of grid points in the image.

Sixteen additional images were taken at random locations in the muscle at 2000× magnification to analyze capillarity. All fibers and capillaries in each image were counted using the unbiased sampling rule outlined in Egginton (1990). Capillary density was calculated as the number of capillaries per image area. Eight images were sufficient to obtain a stable mean for each individual.

Succinate dehydrogenase staining and analysis

Succinate dehydrogenase activity was used as a marker for mitochondria to distinguish and quantify oxidative and glycolytic fiber types. Tissue samples from both the pectoralis and gastrocnemius were coated with embedding medium before being flash frozen in 2-methylbutane that was cooled to near freezing in liquid N_2. These frozen samples were stored in liquid N_2 until transfer to a −80°C freezer. Because liquid N_2 was not available where the common eiders were collected, they were only included in the TEM analyses (see above). Frozen samples were sectioned (10 µm) transverse to muscle fiber length using a Leica CM 1850 Cryostat at −20°C. Sections were dried at room temperature for 2 h, and then incubated in working buffer (2.0 mmol l^{-1} KH_2PO_4, 15.4 mmol l^{-1} Na_2HPO_4, 16.7 mmol l^{-1} sodium succinate, 0.61 mmol l^{-1} nitroblue tetrazolium chloride, 1.3 mmol l^{-1} NaCN) at avian body temperature (41°C) for 1 h under constant rotation. After incubation, sections were rinsed, post-fixed in sucrose formol fixative [160 mmol l^{-1} sucrose, 55 mmol l^{-1} $Ca(NO_3)_2$, 3.3% formaldehyde] for 2 min, and rinsed again. Images were captured using a Leica DM IL inverted microscope. Each image was analyzed for areal density of oxidative and glycolytic fibers (Egginton, 1990; Weibel, 1979) using a grid area of 3000 μm^2. A total of 8–16 images were captured and analyzed depending on the size of the section, which exceeded the minimum of six images that was necessary to reach a stable mean for each individual.

Phylogeny

The phylogeny used in our analyses was adapted from Schell et al. (2023). Briefly, the phylogeny was reconstructed using autosomal double digest restriction-site associated DNA sequencing (ddRAD-seq) data for all species. Sequences were aligned using the pipeline outlined in Lavretsky et al. (2020). The tree was reconstructed using SNAPP (Leaché et al., 2014) in *BEAST v. 2.5.2 (Bouckaert et al., 2014) and run for 100,000,000 iterations with a burn in of 10% and sampling every 1000 iterations to obtain an effective sample size of ≥100.

The original phylogeny published in Schell et al. (2023) contained all species in this study except the common eider. To add in the common eider, we adjusted our phylogeny to match the sea duck topology from Lavretsky et al. (2021), which has the common eider (and other eider species) sister to the long-tailed duck, which in turn are sister to the harlequin duck plus the rest of the sea ducks (Lavretsky et al., 2021, their fig. 4). Preliminary analyses yielded similar results using a phylogeny with actual branch lengths and a phylogeny with all branch lengths set to 1. For uniformity, results of all analyses reported here used our updated phylogeny with all branch lengths set to 1 (Fig. 1).

Dive times

All dive times are reported as mean dive time and maximum dive time, with non-diving species assigned a dive time of 0 s (Table S1). We undertook a comprehensive literature search for each species. Where multiple dive times were reported, we prioritized accounts of wild birds in their natural habitat and studies that reported dive times for multiple species to reduce observer bias (Schell et al., 2023).

Statistical analyses

All data are reported as the mean±s.e.m. Given the close evolutionary relationships of the 17 species studied here, all statistical analyses were conducted in a phylogenetic framework to account for the non-independence of data points (Felsenstein, 1985). Phylogenetic signal in each trait was tested for by measuring Pagel's λ (Pagel, 1999), followed by χ^2 tests to determine whether the value was significantly different from perfect Brownian motion (λ=1).

We first wanted to determine whether muscle phenotypes (mitochondrial volume, proportion of subsarcolemmal mitochondria,

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fiber transverse area, areal density of oxidative and glycolytic fibers, and capillarity) were significantly different between species with different diving habits. Phylogenetic ANOVA accounts for species identity and relatedness in its covariance matrix, making it impossible to test for differences between individual species in a phylogenetic context. Instead, we collapsed species into testable groups of the three clades with varying dive capabilities: sea ducks, pochards, dabblers. All traits were tested using phylogenetic ANOVA (phytools, nsim=1000; Revell, 2012), with Benjamini–Hochberg *post hoc* tests to make pairwise comparisons between each group.

We next wanted to learn whether differences in mitochondrial abundance or capillarity were more highly correlated with dive performance (as reflected by mean dive time) for each species using phylogenetic generalized least squares regression (PGLS). Owing to the highly collinear nature of all mitochondrial variables, we chose average mitochondrial volume density in the muscle as an overall representation of mitochondria in the gastrocnemius, and capillary density as an overall representation of capillarity. Previous work has shown that dive time is correlated with body mass and blood- and muscle-O_2 stores (Elliott et al., 2010; Ponganis, 2015), so we also included those as covariates in the model for a total of five variables: average mitochondrial volume density, capillary density, body mass, hemoglobin [Hb] concentration and myoglobin [Mb] concentration from Schell et al. (2024) (Table 2). We used the AICcmodavg package (https://cran.r-project.org/package=AICcmodavg) for our model selection and based the best model off the lowest AICc, including both the null and global models for completeness. Because the calculation of average muscle mitochondria required data from the fiber-typing analyses using SDH staining, common eider was excluded from this analysis.

RESULTS

Phylogenetic signal

Of all traits studied, five showed phylogenetic signal, with λ values that were not significantly different from 1 (χ^2, P>0.05). These traits were: mitochondrial volume density in glycolytic fibers (λ=0.742, P=0.073), volume density of intermyofibrillar mitochondria in glycolytic fibers (λ=0.828, P=0.111), volume density of subsarcolemmal mitochondria in oxidative fibers (λ=0.896, P=0.122), proportion of subsarcolemmal mitochondria in oxidative fibers (λ=0.908, P=0.200), and the areal density of oxidative fibers in the pectoralis (λ=0.629, P=0.051).

Differences in muscle phenotype between tribes

After accounting for phylogenetic signal, sea ducks were still found to have significantly higher overall mitochondrial volume density in both oxidative (P=0.007) and glycolytic (P=0.007) fibers compared with dabblers (Table 1). In both fiber types, this overall greater mitochondrial volume density was driven by a significantly higher volume density of subsarcolemmal mitochondria (P=0.005 [oxidative], 0.016 [glycolytic]) (Fig. 2). There was a concurrently higher intermyofibrillar mitochondrial volume density in the glycolytic fibers (P=0.033), but not in the oxidative fibers (P=0.217). Correspondingly, the proportion of total mitochondrial volume made up by the subsarcolemmal fraction was significantly higher in sea ducks compared with dabblers in oxidative fibers (P=0.004), but not in glycolytic fibers (P=0.223) (Fig. 3). When the average mitochondrial abundance across the gastrocnemius was calculated, accounting for the variation in the densities and mitochondrial volume between oxidative and glycolytic fibers (see Materials and Methods), sea ducks also had significantly higher abundance compared with dabblers (P=0.002) (Table 1). Pochards were generally intermediate and did not differ significantly from either dabblers or sea ducks (all P>0.05).

Although there was no difference in the areal density of oxidative fibers in the gastrocnemius (P=0.599), sea ducks had a significantly greater areal density of oxidative fibers in the pectoralis compared with the dabblers (P=0.002; Fig. 4, Table 1). Transverse fiber area was not significantly different between tribes, but glycolytic fibers were 1.4–1.7 times larger compared with oxidative fibers across tribes (all P<0.01). Capillary density and the capillary-to-fiber ratio were also not significantly different between tribes (Table 1). However, ordinary least squares regression using all individuals showed that both capillary density and the capillary-to-fiber ratio were positively correlated with the average mitochondrial abundance across the muscle (Fig. 5).

Correlations of muscle phenotype with dive time

The best model correlating muscle phenotypes with dive time using PGLS included average mitochondrial volume density in the gastrocnemius, total body mass and [Mb] in the gastrocnemius (AICc=96.3, Table 2). In this model, [Mb] was the sole significant predictor of dive time (P=0.004), although average mitochondrial volume density was an almost significant predictor (P=0.052). Mitochondrial volume density was included as a predictor in four of the top five models (ΔAICc=3.8–7.1; Table 2).

Table 1. Muscle phenotypes (±s.e.m.) in the gastrocnemius and pectoralis of diving and non-diving ducks

Muscle trait	Sea ducks (*n*=53)	Pochards (*n*=18)	Dabblers (*n*=18)
Glycolytic fiber mitochondrial volume density	0.234±0.009**	0.170±0.015	0.136±0.038
Subsarcolemmal mitochondrial volume density	0.087±0.005*	0.053±0.008	0.038±0.006
Intermyofibrillar mitochondrial volume density	0.147±0.005*	0.117±0.008	0.098±0.009
Oxidative fiber mitochondrial volume density	0.384±0.006**	0.345±0.014	0.288±0.010
Subsarcolemmal mitochondrial volume density	0.182±0.004**	0.153±0.008	0.116±0.007
Intermyofibrillar mitochondrial volume density	0.201±0.003	0.191±0.007	0.172±0.004
Average mitochondrial volume density	0.311±0.007**	0.255±0.013	0.198±0.011
Oxidative fiber transverse area (μm^2)	1216±42	1384±95	1120±83
Glycolytic fiber transverse area (μm^2)	1974±72	1911±86	1957±75
Capillary/fiber ratio	1.67±0.05	1.43±0.09	1.41±0.09
Capillary density (mm^{-2})	1050±40	964±73	784±31
Oxidative fiber areal density	Sea ducks (*n*=61)	Pochards (*n*=24)	Dabblers (*n*=39)
Gastrocnemius (%)	48.8±1.2	49.3±1.9	44.3±1.3
Pectoralis (%)	89.2±0.9**	83.1±0.7	71.7±1.0

All muscle traits include the common eider except estimates of areal density and average mitochondrial volume density. *P<0.05 or **P<0.01 compared with dabblers in phylogenetic ANOVA Benjamini–Hochberg *post hoc* tests. *n*, sample size per tribe.

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Table 2. Results of model selection to determine whether changes in mitochondrial abundance or capillarity were more highly correlated with dive time using AICcmodavg

Model	*K*	AICc	ΔAICc	–lnL	Intercept	Mass	Avg Mito	CD	[Mb] (g)	[Hb]
Avg mito+mass+[Mb]	5	96.27*	0	–39.39	–28.4±8.9 (0.01)	0.002±0.004 (0.64)	86.5±36.1 (0.04)	–	3.2±0.9 (0.005)	–
Avg mito	3	100.07	3.80	–45.83	–26.3±12.1 (0.051)	–	151.2±45.7 (0.006)	–	–	–
Avg mito+mass+[Mb]+[Hb]	6	100.25	3.98	–38.12	–56.5±23.0 (0.04)	0.0006±0.004 (0.88)	72.6±36.4 (0.08)	–	3.1±0.8 (0.006)	2.5±1.7 (0.22)
CD+mass+[Mb]	5	100.44	4.17	–41.47	–17.7±9.3 (0.09)	0.00001±0.005 (0.98)	–	0.007±0.008 (0.36)	4.2±0.9 (0.001)	–
Avg mito+mass	4	103.37	7.10	–45.46	–31.9±12.8 (0.03)	0.007±0.006 (0.26)	153.3±45.0 (0.006)	–	–	–
CD+mass+[Mb]+[Hb]	6	103.98	7.71	–39.99	–58.2±26.9 (0.06)	–0.001±0.005 (0.78)	–	0.006±0.007 (0.44)	3.8±0.9 (0.002)	3.0±1.9 (0.15)
Null	2	106.56	10.29	–50.74	–	–	–	–	–	–
Avg+mass+[Hb]	5	107.21	10.93	–44.85	–68.1±33.8 (0.07)	0.005±0.006 (0.43)	132.1±48.0 (0.02)	–	–	2.9±2.5 (0.27)
CD	3	108.66	12.38	–50.13	1.3±13.3 (0.92)	–	–	0.01±0.01 (0.45)	–	–
CD+mass	4	112.21	15.93	–49.88	–3.0±14.7 (0.84)	0.006±0.008 (0.48)	–	0.009±0.01 (0.47)	–	–
Global	8	117.85	21.57	–36.53	–32.4±35.0 (0.38)	0.003±0.005 (0.57)	80.2±40.4 (0.09)	–0.0004±0.008 (0.96)	3.74±1.1 (0.01)	2.97±1.9 (0.17)

Values are means±s.e.m. with the associated *P*-value in parentheses. The best model with the lowest AICc is placed at the top. *K*, number of model parameters; avg mito, average mitochondrial volume density in the gastrocnemius; mass, total body mass; CD, capillary density; [Mb], myoglobin concentration in the gastrocnemius; [Hb], blood hemoglobin concentration; –lnL, log likelihood of each model.

DISCUSSION

The ability of air-breathing vertebrates to forage successfully underwater and sustain aerobic locomotion without breathing necessitates multiple specializations in O_2 storage, delivery and utilization. Our results suggest that a higher oxidative capacity in the swimming muscles helps enable longer dive times in these small-bodied diving ducks (Fig. 2, Table 1), potentially by improving endurance capacity and/or the extraction and utilization of O_2 from blood and tissue O_2 stores. Comparisons across three clades of diving and non-diving ducks showed that longer dive times in the diving ducks are correlated with higher mitochondrial volume densities, with much higher densities seen in the subsarcolemmal fraction in both oxidative and glycolytic fibers of the gastrocnemius, as well as a higher areal density of oxidative fibers in the pectoralis. These changes provide evidence that diving ducks rely on a greater muscle oxidative capacity to fuel the ATP demands of foraging dives, likely helping avoid anaerobic ATP production.

Mitochondrial volume density and distribution

The volume densities of mitochondria reported here are some of the highest reported for diving animals (Table 1). Previous work has shown that mitochondrial volume scales inversely with body mass (Hoppeler and Weibel, 2000; Mathieu et al., 1981), which could explain why our mitochondrial volume densities for ducks ranging in body mass from 400 g to 3 kg are much higher than those found in another diving bird, the 25 kg emperor penguin (*Aptenodytes forsteri*) (Ponganis et al., 1997a). The volume densities of mitochondria here are much higher than the previously reported value of 0.085 in the gastrocnemius of the tufted duck (Turner and Butler, 1988). However, this previous study did not distinguish oxidative and glycolytic fibers, and the high 25,000× magnification used would have precluded quantification of volume density across entire fibers, which requires much lower magnifications for accurate quantification.

Mitochondrial volume density was greater in both oxidative and glycolytic fibers of sea ducks (Fig. 2), and mitochondrial volume density was a predictor of dive capabilities in four of the top five correlative models (Table 2). This suggests that the best divers have the most oxidative muscle and have high capacities for aerobic metabolism to support sustained locomotion. However, the extent to which this high oxidative capacity of locomotory muscles is realized throughout the dive is unclear. It is unknown in these species the rate

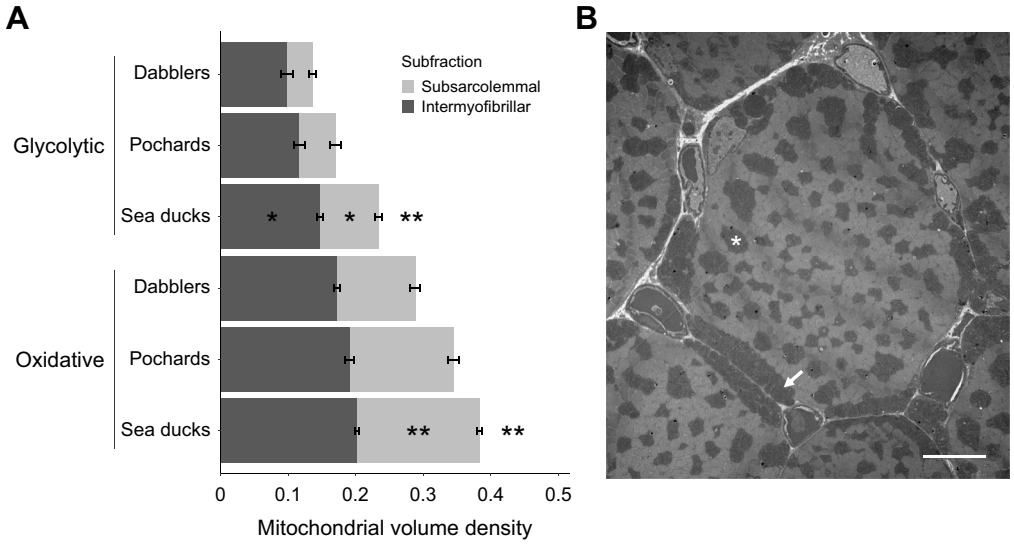

Fig. 2. Mitochondrial volume density and distribution in both oxidative and glycolytic fibers across three tribes of ducks: sea ducks (diving, *n*=53), pochards (diving, *n*=18) and dabblers (non-diving, *n*=18). (A) The overall higher mitochondrial volume density seen in the sea ducks is due to concurrently high volume densities of the subsarcolemmal fraction of mitochondria in both fiber types (asterisks in the light gray bars), and higher intermyofibrillar volume density in glycolytic fibers (asterisks in the dark gray bars). **P*<0.05 or ***P*<0.01 compared with dabblers in phylogenetic ANOVA Benjamini–Hochberg *post hoc* tests. (B) Representative TEM image of an oxidative fiber from a greater scaup (*Aythya marila*) showing subsarcolemmal (arrow) and intermyofibrillar (asterisk) mitochondria. Scale bar: 8 µm.

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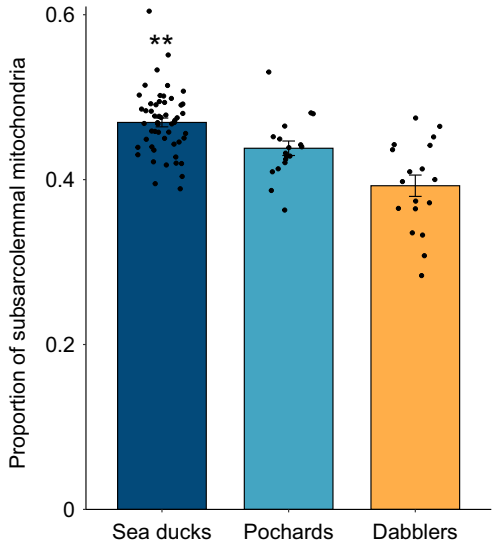

Fig. 3. The proportion of mitochondria in the subsarcolemmal subfraction of oxidative fibers is greater in the sea ducks (diving, *n*=53) compared with the dabblers (non-diving, *n*=18), with the pochards (diving, *n*=18) intermediate between the two. Phylogenetic ANOVA (*P*=0.002) with Benjamini–Hochberg *post hoc* tests **P<0.01 compared with dabblers.

at which blood- and muscle-O_2 stores decrease throughout the duration of a normal dive, which would influence whether working leg muscles remain aerobic later in a dive. However, the preferential enrichment of subsarcolemmal mitochondria may facilitate the extraction of blood-O_2 stores during the later stages of a dive because this mitochondrial fraction is nearest to capillaries. Indeed, some birds and mammals adapted to high-altitude hypoxia have also evolved a greater abundance of subsarcolemmal mitochondria in muscle (Mahalingam et al., 2017; Scott et al., 2009). This high abundance of subsarcolemmal mitochondria likely acts as a large O_2 sink that would facilitate O_2 diffusion into the cell by maintaining a strong gradient for O_2 diffusion, even as blood-O_2 stores are depleted (Ponganis et al., 2007).

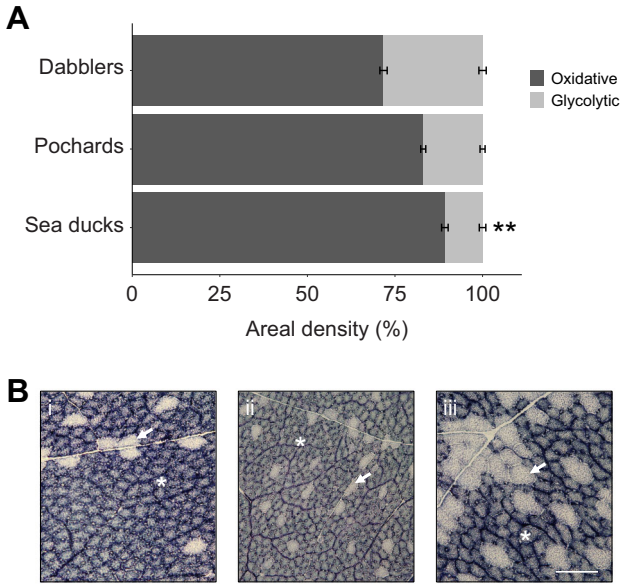

Fig. 4. The areal density of oxidative and glycolytic fibers in the pectoralis muscle in three tribes of ducks: sea ducks (diving, *n*=61), pochards (diving, *n*=24) and dabblers (non-diving, *n*=39). (A) Areal density of oxidative fibers (dark gray) was significantly higher, and glycolytic fibers (light gray) was significantly lower, in the sea ducks compared with the dabblers. Phylogenetic ANOVA (*P*=0.005) with Benjamini–Hochberg *post hoc* tests (**P<0.01). (B) Succinate dehydrogenase staining of pectoralis muscle in a (i) a sea duck (common merganser, *Mergus merganser*), (ii) a pochard (ring-neck duck, *Aythya collaris*) and (iii) a dabbler (northern shoveler, *Anas clypeata*), showing the changes in areal density of oxidative (asterisk) and glycolytic (arrow) fibers between tribes. Scale bar: 100 µm.

The observed differences in the density of subsarcolemmal mitochondria in sea ducks compared to dabblers contrasts findings in diving pinnipeds. In these diving mammals, upwards of 70–90% of mitochondria are located in the intermyofibrillar fraction (Kanatous et al., 1999), far greater than the 50–60% observed here in ducks. This characteristic of pinnipeds has been interpreted as a strategy to facilitate utilization of the significant O_2 stores bound to Mb throughout the sarcoplasm of locomotory muscle fibers (Davis et al., 2004). It has been hypothesized that the strong dive response of bradycardia and peripheral vasoconstriction may limit muscle blood flow and lead to reductions in metabolism while diving, which allows for O_2 savings in non-swimming muscles and tissues, as well as inducing modest tissue hypoxemia that mobilizes O_2 from Mb-bound O_2 stores (Davis and Kanatous, 1999; Davis and Williams, 2012; Favilla and Costa, 2020). Although the sea ducks studied here also have elevated [Mb] in the gastrocnemius (Schell et al., 2024), and [Mb] was a significant predictor of dive time (Table 2), they are also known to increase blood flow to the leg muscles by five-fold during voluntary diving (Bevan and Butler, 1992). It is possible that diving ducks, which have shorter duration, highly energetic dives, rely more heavily on blood-O_2 stores owing to a more pronounced heart rate response to locomotion as a strategy for maximizing O_2 extraction during dives.

Whereas a higher concentration of subsarcolemmal mitochondria is likely beneficial for accessing bloodborne O_2 and metabolic fuels, large aggregations of mitochondria at the edge of the cell could increase diffusion distances for ATP (Kinsey et al., 2007). However, subsarcolemmal mitochondria are electrically connected to intermyofibrillar mitochondria in a reticulum, such that the former prioritize generation of proton-motive force whereas the latter prioritize ATP generation closer to where contraction occurs, which would help overcome the issue of diffusion distance (Glancy et al., 2015). This has been shown to be associated with functional differences between subsarcolemmal and intermyofibrillar mitochondria, with the former having lower ATP synthase activity (Cogswell et al., 1993; Dawson and Scott, 2022). Thus, the interconnectedness of the mitochondrial subfractions assures that preferential enrichment of subsarcolemmal mitochondria may not constrain ATP supply to the interior of the muscle. In our prior study, ATP synthase activity in the gastrocnemius was not different between the three tribes (Schell et al., 2023), suggesting that the overall capacity for ATP generation in the muscle is preserved.

Muscle capillarity

The overall greater mitochondrial volume densities seen in sea ducks were positively correlated with both capillary density and the capillary-to-fiber ratio (Fig. 5). Our results are consistent with previous work showing that capillarity scales with mitochondrial volume (Conley et al., 1987; Hoppeler et al., 1981), although mammalian divers have lower capillarity than expected by this relationship (Kanatous et al., 1999). The correlation seen here suggests that blood-O_2 stores support muscle O_2 demands across these species. Given that diving ducks strongly perfuse the leg

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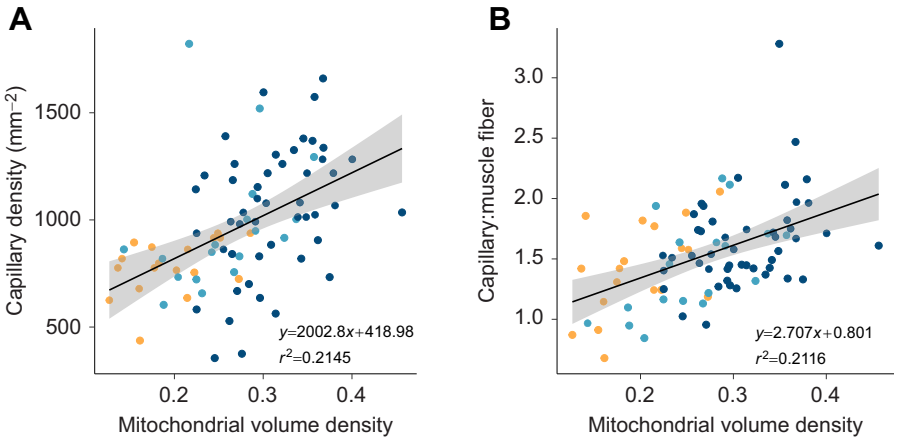

Fig. 5. Both capillary density and capillary to muscle fiber ratio were significantly correlated with the average mitochondrial volume density across the gastrocnemius for 16 species of ducks. (A) Capillary density (mm^{-2}) and (B) capillary:muscle fiber ratio. Best fit line determined by ordinary least squares regression, with 95% confidence interval shown by gray shading (P<0.001). Tribes are indicated by color: sea ducks, dark blue; pochards, teal; and dabblers, gold.

muscles while diving (Bevan and Butler, 1992), and that both sea ducks and pochards have a higher blood [Hb] compared with the dabblers (Schell et al., 2024), blood flowing through the capillary beds should provide a significant source of O_2 to fuel muscle metabolism during dives (Hedrick and Duffield, 1991).

Oxidative capacity in the flight muscle

Although there was no difference in the areal density of oxidative fibers in the gastrocnemius, there was a striking difference in the pectoralis between the sea ducks and the dabblers (Fig. 4). The pectoralis of the long-tailed duck, in particular, was composed of 99% oxidative fibers (Table S2). The phenotype of the pectoralis impacts the biomechanics of flight, with the fast-contracting glycolytic fibers being most important in generating short bursts of power during takeoff, while the more fatigue-resistant oxidative fibers are most important during sustained flight (Norberg, 2012). Dabbling and diving ducks differ in how they take off from the water, with dabblers 'rocketing' vertically off the surface whereas diving ducks tend to run across the surface to gain momentum and lift. Although these differences have been previously attributed to the evolution of different morphologies associated with divergent feeding strategies (i.e. larger mass of the pectoralis in dabbling ducks, hind limbs placed farther to the posterior in diving ducks), our results here suggest that underlying differences in muscle phenotype may also play a role (Bethke and Thomas, 1988; Raikow, 1973). The higher proportion of glycolytic fibers in the pectoralis of dabblers should allow for them to generate the short burst of energy required for a vertical takeoff. The sea ducks and pochards may not be able to generate the muscle forces required for vertical takeoff with the lower proportion of glycolytic fibers present, leading to long takeoff runs supported primarily by oxidative fibers.

Conclusions

The locomotory muscles of ducks appear to be specialized to support high rates of aerobic metabolism during locomotion. In the gastrocnemius, sea ducks had greater mitochondrial volumes than dabblers, particularly driven by the preferential proliferation of subsarcolemmal mitochondria, which was correlated with higher capillary density and capillary-to-fiber ratio. Together, these findings emphasize the vital importance of both blood- and muscle-O_2 stores for diving and that locomotory muscles are restructured to enhance the utilization of these O_2 stores during natural dives. Our study highlights several key adaptations that support dive performance across this understudied group of diving birds. Future directions might include the studies of intra-individual variation across the annual cycle as affected by shifts in diet, migration chronology, timing of breeding, or exercise performance.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: E.R.S., G.R.S., N.J.D., K.G.M.; Methodology: G.R.S.; Validation: E.R.S.; Formal analysis: E.R.S.; Investigation: E.R.S.; Resources: G.R.S., K.W., K.G.M.; Data curation: E.R.S.; Writing - original draft: E.R.S.; Writing - review & editing: G.R.S., N.J.D., K.W., K.G.M.; Visualization: E.R.S.; Supervision: G.R.S., K.G.M.; Project administration: E.R.S.; Funding acquisition: K.W., K.G.M.

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Data availability

Data are available from duckDNA.org: https://duckdna.org/data/jeb247550/.

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