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RESEARCH ARTICLE

Respiratory mechanics of eleven avian species resident at high and low altitude

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ABSTRACT

The metabolic cost of breathing at rest has never been successfully measured in birds, but has been hypothesized to be higher than in mammals of a similar size because of the rocking motion of the avian sternum being encumbered by the pectoral flight muscles. To measure the cost and work of breathing, and to investigate whether species resident at high altitude exhibit morphological or mechanical changes that alter the work of breathing, we studied 11 species of waterfowl: five from high altitudes (>3000 m) in Perú, and six from low altitudes in Oregon, USA. Birds were anesthetized and mechanically ventilated in sternal recumbency with known tidal volumes and breathing frequencies. The work done by the ventilator was measured, and these values were applied to the combinations of tidal volumes and breathing frequencies used by the birds to breathe at rest. We found the respiratory system of high-altitude species to be of a similar size, but consistently more compliant than that of low-altitude sister taxa, although this did not translate to a significantly reduced work of breathing. The metabolic cost of breathing was estimated to be between 1 and 3% of basal metabolic rate, as low or lower than estimates for other groups of tetrapods.

KEY WORDS: Avian respiratory system, Compliance, Hypoxia, Pulmonary mechanics, Waterfowl, Work of breathing

INTRODUCTION

For animals living at high altitude, where oxygen supply is limited, it is not always possible to decrease oxygen demand, as metabolically expensive but vital tasks such as predator evasion, hunting or foraging, migration and reproduction must still be accomplished. Animals must therefore match oxygen demand by increasing oxygen supply, and some adaptive changes in the oxygen transport cascade, such as increased hemoglobin–oxygen affinity, have been well studied (Hochachka, 1985; Monge and Leon-Velarde, 1991; Storz et al., 2010; Storz, 2016). Increasing the first step in the oxygen transport cascade, ventilation, is often the first response when oxygen demand outstrips supply (Powell et al., 1998). Increasing ventilation can be accomplished either by

increasing the volume of the breath (tidal volume, V_t) or the frequency of respiration (f_R) such that:

$$V_{tot} = f_R \times V_t, \tag{1}$$

where V_{tot} is total ventilation. In vertebrates such as fish, which can bring nearly all the 'inhaled' respiratory medium into direct contact with the gas exchange surface, increasing f_R is an equally effective strategy as increasing V_t. However, for tetrapods, the dead space volume (DS) of the respiratory system must be taken into account, such that:

$$\dot{V}_{eff} = f_R \times (V_t - DS). \tag{2}$$

The dead space is the volume of respiratory medium that the animal expends energy to move from the environment into the respiratory system, but which never contacts the gas exchange surface and so does not contribute to oxygen loading. In the case of sauropsids, who use active expiration, this volume is not only actively moved into the animal but must also be actively pushed out to prepare for the next breath, thus doubling the cost per unit volume compared with animals using passive expiration (e.g. mammals at rest; Codd et al., 2005). The volume of respiratory medium that contacts the respiratory surface per minute is called the effective ventilation ($\dot{V}_{eff}$); because dead space volume is a constant, increasing V_t increases $\dot{V}_{eff}$ more than an equivalent increase of f_R.

This more effective strategy of increasing V_t over f_R is generally more metabolically expensive, however, because the work to overcome the elastic forces of the chest wall and respiratory system increases exponentially with increasing V_t, whereas resistive forces increase only linearly with f_R (as measured in a turtle; Vitalis and Milsom, 1986). Despite this, high-altitude avian species such as the bar-headed goose (*Anser indicus*) and Andean goose (*Chloephaga melanoptera*) have been shown to preferentially increase V_t over f_R (Scott and Milsom, 2007; Lague et al., 2016; S.L.L., B.A.C., L.A., Y. Zhong, A. P. Farrell, K.G.M., Y. Wang and W.K.M., unpublished data). We wondered whether this deeper, slower breathing pattern might be found in other high-altitude species, and whether the mechanical design of the respiratory systems of high-altitude species might be altered to reduce the metabolic cost of potentially employing this higher tidal volume strategy. To investigate this, we compared the mechanics of the respiratory system and the work of breathing in 11 species of waterfowl from two sites: a low- and a high-altitude site (see Table 1 for a list of species). Eight of the species selected were pairs of sister taxa in which one species or subspecies is found primarily at high altitude and the other at low altitude. This allowed us to make four phylogenetically independent comparisons of the effects of altitude on respiratory mechanics.

With these four independent comparisons on the effects of altitude on respiratory mechanics, we wanted to answer four primary questions: (1) are there morphological or mechanical differences in

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Table 1. List of species compared in this study and values of respiratory volumes, static compliance, lung mass and heart mass

Species	Abbreviation	Body mass (g)	Vital capacity (ml)	Vital capacity (ml kg^{-1})	Inspiratory capacity (ml kg^{-1})	Expiratory capacity (ml kg^{-1})	Extra-pulmonary airway volume (ml)	Static compliance (ml cmH$_2$O^{-1})	Static compliance (% vital capacity cmH$_2$O^{-1})	Mass (g kg^{-1}) Total lung	Whole heart	Right ventricle	Left ventricle
Highland													
Puna teal *Anas puna*	PT	396±15	157±4	399±14	246±12	154±10	1.9±0.3	8.70±0.23	5.56±0.13	16.7±0.9	9.4±0.6	1.5±0.2	6.1±0.4
Speckled teal *Anas flavirostris oxyptera*	ST	378±9*	132±3***	350±8	224±9	126±10	1.3±0.3	7.09±0.24**	5.47±0.22*	16.3±1.6	12.9±0.6	2.3±0.2	7.7±0.3
Ruddy duck *Oxyura jamaicensis ferruginea*	RD	773±51***	193±6***	260±19*	194±12	67±10**	1.6±0.1	9.71±0.74**	4.75±0.25	16.1±1.3	9.5±0.5	1.3±0.1	5.8±0.4*
Yellow-billed pintail *Anas georgica*	YBP	618±20***	234±6	373±7**	236±8**	137±4	2.8±0.4***	12.9±0.60	5.51±0.26	14.6±1.3	10.4±0.6	2.0±0.1	6.2±0.5*
Cinnamon teal *Anas cyanoptera orinomus*	CT	440±13**	159±4***	360±13	240±13	121±8	2.6±0.3	8.48±0.30***	5.36±0.22*	14.4±1.2	11±0.6	1.9±0.2	6.6±0.4
Lowland													
Green-winged teal *Anas crecca*	GT	283±10	94±3	331±18	206±8	125±14	1.3±0.3	4.41±0.31	4.68±0.24	17.1±1.5	12.3±2	2.9±0.4	9.0±2
Lowland ruddy duck *Oxyura jamaicensis jamaicensis*	LRD	476±32	153±7	329±28	217±16	112±17	1.4±0.2	6.97±0.69	4.49±0.24	18.1±1.3	9.7±0.9	2.1±0.5	7.3±0.5
Northern pintail *Anas acuta*	NP	857±12	249±10	291±12	183±9	108±7	4.9±0.2	11.8±0.83	4.72±0.15	13.8±1.9	10.7±0.1	2.2±0.3	8.0±0.3
Lowland cinnamon teal *Anas cyanoptera septentrionalium*	LCT	300±13	104±4	350±21	218±14	132±16	1.8±0.2	4.87±0.32	4.69±0.25	16.7±2.4	9.2±0.4	1.9±0.3	6.8±0.2
Mallard duck *Anas platyrhynchos*	MD	932±21	330±19	359±23	248±20	111±5	6.0±0.1	14.3±1.27	4.34±0.23	13.5±1.7	7.4±0.4	1.3±0.3	5.9±0.2
Gadwall *Anas strepera*	GW	766±40	284±10	372±16	226±16	147±11	5.4±0.4	11.5±0.68	4.02±0.12	13.8±0.6	10.5±3	2.1±0.6	8.1±2

Asterisks indicate significant differences between sister taxa using a two-way ANOVA with *post hoc* Tukey tests (*$P<0.05$; **$P<0.01$; ***$P<0.001$).

the respiratory system associated with colonization of high-altitude environments; (2) are there relative benefits of increasing tidal volume over breathing frequency; (3) what is the actual cost of breathing for these birds *in vivo*; and (4) do these birds use an energetically 'optimal' combination of tidal volume and breathing frequency?

MATERIALS AND METHODS

High-altitude species were collected from Lake Titicaca National Reserve, Puno, Perú, at 3812 m above sea level. Low-altitude species were collected from either Summer Lake Wildlife area at 1260 m or Malheur National Wildlife Refuge at 1256 m in Oregon, USA. Species collected at the high-altitude site were the puna teal (*Anas puna*; *n*=6), speckled teal (*Anas flavirostris oxyptera*; *n*=6), Andean ruddy duck (*Oxyura jamaicensis ferruginea*; *n*=11), yellow-billed pintail (*Anas georgica*; *n*=6) and cinnamon teal (*Anas cyanoptera orinomus*; *n*=8). Species collected at the low-altitude site were the American green-winged teal (*Anas crecca*; *n*=8), ruddy duck (*Oxyura jamaicensis jamaicensis*; *n*=6), northern pintail (*Anas acuta*; *n*=6), cinnamon teal (*Anas cyanoptera septentrionalium*; *n*=8), mallard (*Anas platyrhynchos*; *n*=6) and gadwall (*Anas strepera*; *n*=6). See Fig. 1 for the phylogenetic relationships among these species (Gonzalez et al., 2009).

The five highland species vary in the evolutionary time since they split with their lowland sister taxa and radiated to altitude. The highland puna teal and speckled teal are the most divergent from their lowland relatives: the lowland silver teal (*Anas versicolor*; not studied here) and the lowland green-winged teal. The highland cinnamon teal and ruddy duck are considered separate subspecies from their lowland sister taxa, the cinnamon teal being the most recently diverged of these sister taxa. Some gene flow still exists between the highland yellow-billed pintails and yellow-billed pintails found at low-altitude sites in South America (McCracken et al., 2009b). In this study, we also include the mallard and gadwall, lowland species who have no high-altitude sister taxa. All of these species, except the ruddy ducks, are considered to be dabbling ducks (genus *Anas*), meaning that they forage primarily while on the surface of the water. The ruddy ducks are stiff-tailed diving ducks and the only representatives in this study of the genus *Oxyura* (see Table 1 and Fig. 1).

Experimental procedures were performed according to University of British Columbia Animal Care Committee protocols
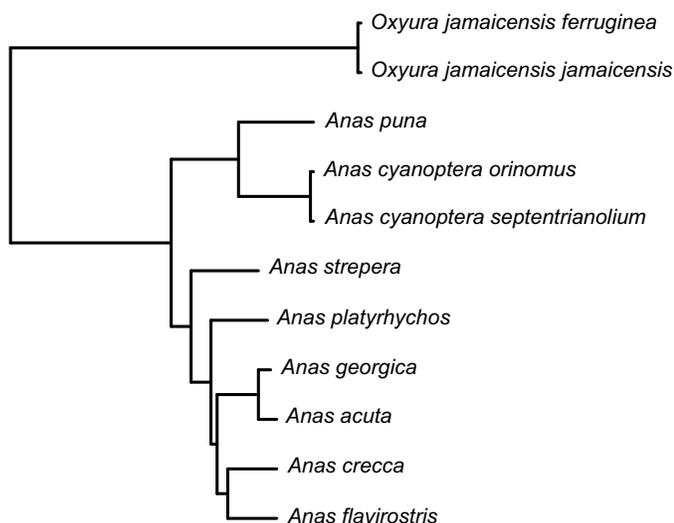
A12-0013 and A16-0019 under the guidelines of the Canadian Council on Animal Care. Wild animals were collected in accordance with the Ministerio del Ambiente del Peru (004-2014-SERNANP-DGANP-RNT/J), Ministerio de Agricultura del Peru (RD 169-2014-MINAGRI-DGFFS/DGEFFS), US Fish & Wildlife Service Migratory Bird Treaty Act (MB68890B-0) and Oregon Department of Fish and Wildlife (Scientific Taking Permit 101-15).

Birds were captured at night and held in small animal cages with access to food and water until the morning. They were then anesthetized with intravenous propofol as described by Mulcahy (2014), intubated and connected to a constant-volume ventilator. Birds were hyperventilated until the spontaneous drive to breathe was lost. They were then ventilated sequentially with at least three volumes (11.55, 17.33, 23.11, 28.9 or 34.68 ml; these numbers were determined by the dimensions of the ventilator) and up to five frequencies at each volume (20, 30, 40, 50 and 60 min^{-1}). Tracheal pressure was not permitted to go above 30 cm H_2O. Pressure at the trachea was measured with a pressure transducer calibrated with a glass manometer, and flow was measured with a differential pressure transducer connected to either side of a pneumotachograph positioned between the intubation tube and the respirator. The flow trace was integrated to give tidal volume. Once dynamic data were collected, the birds were again hyperventilated, disconnected from the ventilator, and a 100 ml glass syringe was used to inflate and then deflate the respiratory system in a step-wise manner (10–20 ml per step) from functional residual capacity (FRC; lungs open to atmospheric pressure) to 30 cmH$_2$O, back to FRC, to −30 cmH$_2$O, and then back to FRC again. This was done two to three times. At the end of experiments, the endotracheal tubes were ventilated with the same volumes and frequencies. Data were recorded with a WinDaq Data Acquisition system at a sample rate of 60 Hz.

Birds were then either allowed to recover and released, or euthanized with an overdose of intravenous propofol (all birds in Perú were euthanized; three individuals of each species in Oregon were euthanized in accordance with our collection permits). Lungs, hearts and tracheas were dissected from euthanized birds. Lung mass, heart mass and extra-pulmonary airway volume were measured. The extra-pulmonary airway volume was used as an estimate of anatomical dead space volume.

All experiments were conducted in the field at the altitudes at which the birds were collected. Thus it is possible that we overestimate the compliance and underestimate the work values in the high-altitude species owing to hypobaric conditions at the high-altitude site. To correct for this, comparisons were made at volumes expressed as a percent of the vital capacities measured at each site. Vital capacity was measured as the volume change between respiratory pressures of 30 and −30 cmH$_2$O. We also normalized values to vital capacity because we were interested in the differences in the mechanics of the respiratory systems independent of differences in body size. Volumes were determined by the dimensions of the pump and therefore are expressed here as atmospheric temperature pressure dry (ATPD).

Static compliance was measured as the steepest slope of the volume and pressure curve under conditions of zero flow. As respiratory system hysteresis was present, the static compliance data are presented from the deflation curve. We also calculated static compliance as the change in volume expressed as a percent of vital capacity for a given change in pressure.

To analyze dynamic data, flow was integrated to produce volume, and volume was plotted against pressure as pressure–volume loops. Dynamic compliance was measured as the slope of the line between the two points of zero flow (minimum and maximum volume).

Fig. 1. Phylogeny of the species compared in this study. Adapted from Gonzalez et al. (2009).

Work to overcome flow resistance (resistive work) was measured as the area between this line and the inflation curve. The work to overcome elastic forces (elastic work) was measured as the area bounded by the compliance line, the y-axis and the line described by: y=maximum volume.

The resistive work to move the air through the endotracheal tube alone was subtracted from total resistive work. Flow resistive and elastic work sum to total work per breath. Resistance was calculated as the difference in pressure between two points of isovolume, divided by the flow. Tau (τ), the time constant of the system, is the product of resistance and compliance.

For measurements of dynamic mechanics, we made comparisons at the same combination of f_R/V_t for all species (approximately the average value derived for all species breathing ambient O_2), with values of V_t expressed as % vital capacity to compare the mechanical properties of individual respiratory systems independent of size. *In vivo*, each species used a different combination of f_R/V_t when breathing ambient air and also increased ventilation with different combinations in hypoxia. Therefore, to compare the mechanics between species *in vivo*, we also estimated work and efficacy at the f_R/V_t combinations used by each species while breathing 13 and 6 kPa O_2. These values of f_R/V_t were taken from Ivy et al. (C.M.I., S.L.L., J.M.Y., B.A.C., L.A., R.C., N.J.D., P.B.F., K.G.M., W.K.M. and G.R.S., unpublished data). We chose 13 kPa O_2 because it was the partial pressure of oxygen (P_{O_2}) at our high-altitude site, and the low-altitude species used a very similar combination of f_R/V_t when breathing 13 and 18 kPa O_2, the P_{O_2} at the low-altitude site (see Fig. 3). We chose 6 kPa O_2 for hypoxia as it was the lowest common P_{O_2} for which data had been collected for these species (C.M.I., S.L.L., J.M.Y., B.A.C., L.A., R.C., N.J.D., P.B.F., K.G.M., W.K.M., and G.R.S., unpublished data). Basal metabolic rates were estimated from the rate of oxygen consumption and CO_2 production measured at these levels of ambient oxygen, also collected by Ivy et al. (C.M.I., S.L.L., J.M.Y., B.A.C., L.A., R.C., N.J.D., P.B.F., K.G.M., W.K.M. and G.R.S., unpublished data).

Data were analyzed using LabChart software from ADInstruments, and graphs and statistics were generated using Origin® 2016. Compliance, τ, elastic work and resistive work were plotted versus tidal volume, and frequency isopleths were produced as linear fits to this data (see Table S1). The equations of these lines were then used to estimate values of the variables at desired combinations of tidal volume and breathing frequency. The effects of lineage and altitude on measured variables were compared among sister taxa pairs (excluding the puna teal, mallard and gadwall) using two-way ANOVAs with *post hoc* Tukey tests (significance level $P<0.05$). We also compared high- versus low-altitude taxa using a paired lineage test.

RESULTS
Respiratory system morphology and static mechanics
The species compared had a wide range of body masses (from roughly 0.3 to 1 kg) and corresponding vital capacities (R^2=0.83). The high-altitude species as a group had significantly higher body masses (Table 1; on average 552±16 g versus 479±16 g; two-way ANOVA; P=0.002) and significantly larger vital capacities (179±3 ml versus 150±3 ml; P<0.001). However, when vital capacity was normalized to body mass, there was no significant effect of altitude (P=0.45). The inspiratory reserve volume accounted for 61–69% of total vital capacity in low-altitude species and 62–75% in high-altitude species. There was no significant effect of altitude on extra-pulmonary airway volume (P=0.19), lung mass when normalized to body mass (P=0.35), or heart mass (P=0.43), and no significant effect of altitude on any morphometric parameter

when compared using a paired lineage test. It was common for one lung to be larger than the other in any individual bird, but whether it was the right or left lung was inconsistent between individuals, and in some birds both lungs were of a similar size.

Static compliance is expressed in ml cmH$_2$O^{-1} (Fig. 2A) and also as the change in volume expressed as a percent of vital capacity (% vital capacity cmH$_2$O^{-1}; Fig. 2B). Expressed as ml cmH$_2$O^{-1}, static compliance in the highland species was significantly greater than in lowland species (two-way ANOVA, P<0.001; paired lineage test, P=0.017). This was largely because they had larger vital capacities (Fig. 2A). The slopes of the static compliance curves were directly proportional to the size of the respiratory system (Fig. 2C; R^2=0.68). When normalized to the size of the respiratory system, a significant effect of altitude on compliance remained (two-way ANOVA, P<0.001; paired lineage test, P=0.015). This was particularly due to an increased compliance below FRC. Among sister taxa, the highland speckled teal and cinnamon teal were significantly more compliant than the lowland green-winged teal and cinnamon teal (P=0.031 and P=0.049, respectively).

Cost and benefit of increasing tidal volume
Dynamic mechanics vary with V_t/f_R, so we calculated values for all of the dynamic variables in each species when breathing at equal tidal volumes (expressed as % of vital capacity) and frequencies in order to compare the mechanics of the respiratory system independent of breathing strategy. When V_t/f_R values were equal, dynamic compliance was lower for the highland ruddy duck and cinnamon teal compared with their lowland sister species, higher for the highland yellow-billed pintail, and inconsistent for the highland speckled teal (Table 2). The time constant also did not differ consistently based on altitude. Resistance at equal V_t/f_R was generally lower or did not significantly differ for the highland species, excluding the ruddy duck. As a result, the total work of breathing (power output) did not differ significantly based on altitude (P=0.69), and in some cases was higher in the highland birds because of decreases in dynamic compliance especially at high ventilation rates (Table 3). In the case of the ruddy ducks, the highland group had both higher resistance and lower compliance than the lowland group, and this resulted in higher work of breathing for the highland ruddy ducks (Tables 2, 3).

Our calculations suggest that doubling V_t would be more expensive than doubling f_R for all species except the highland speckled and cinnamon teals (Table 3). When we calculated the level of effective ventilation per unit energy spent (efficacy), we found the three smallest species (green-winged teal, speckled teal and lowland cinnamon teal) were the most efficacious, especially as ventilation increased (Table 3). The highland birds were significantly less efficacious than their lowland sister taxa at high ventilation rates (P<0.001).

Dynamic mechanics and work of breathing *in vivo*
We calculated the dynamic compliance and the time constant for each species from our data using the *in vivo* breathing patterns measured by Ivy et al. (C.M.I., S.L.L., J.M.Y., B.A.C., L.A., R.C., N.J.D., P.B.F., K.G.M., W.K.M. and G.R.S., unpublished data) and saw no consistent differences between species. As expected, both compliance and the time constant were reduced when birds were breathing 6 kPa O_2 compared with 13 kPa O_2, owing to the increase in ventilation (data not shown).

Not surprisingly, the power (work per minute) required to produce these breathing patterns was significantly higher, and the amount of O_2 delivered to the respiratory exchange surfaces tended
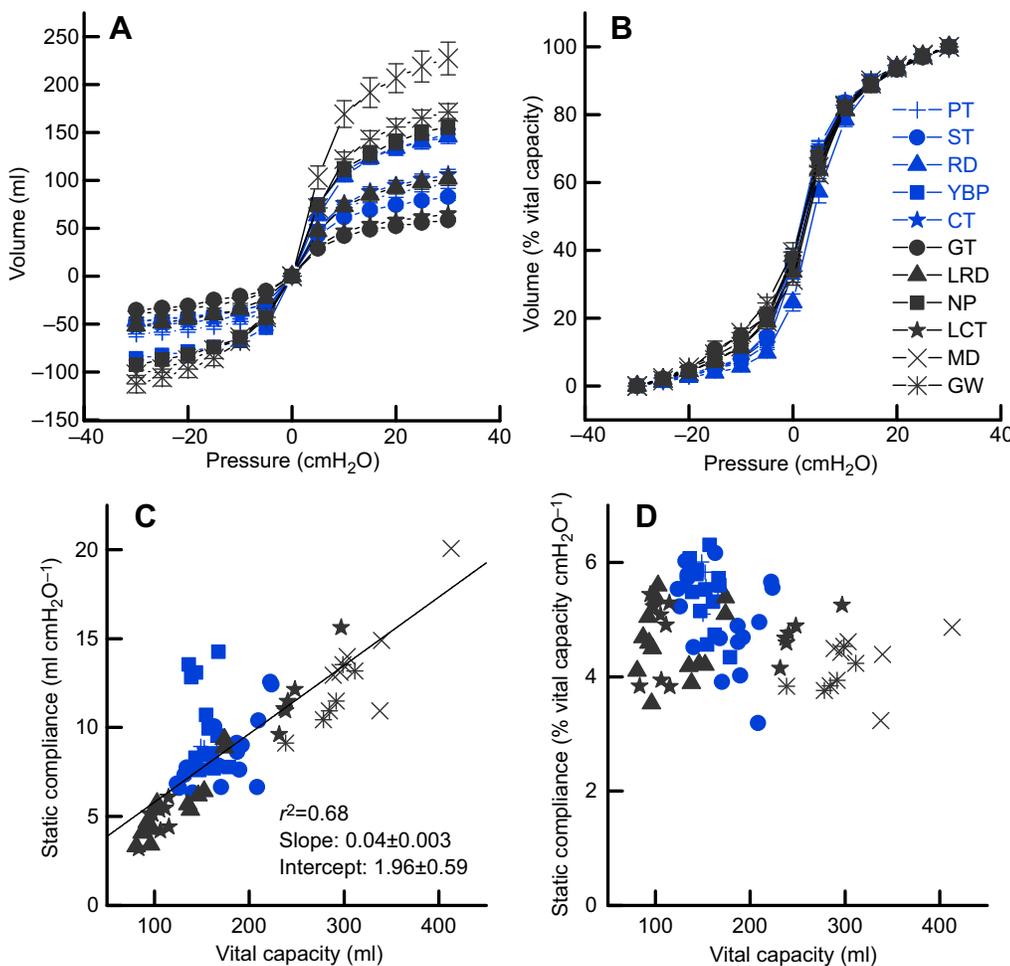
Fig. 2. Static compliance curves and slope values measured at the steepest part of the curves. (A,B) Pressure–volume curves of the intact respiratory system and (C,D) static compliance versus vital capacity. (A) Values are plotted as the change in volume for a given change in pressure. (B) Values are plotted as the change in percent of vital capacity for a given change in pressure. (C) Static compliance is directly proportional to total vital capacity before normalization. (D) When normalized to vital capacity, static compliance is a measurement of the stiffness of the system independent of total respiratory system size. High-altitude species are in blue; low-altitude species are in black. Sister taxa are indicated by shape. See Table 1 for species abbreviations.

to be lower, when the birds were breathing 6 kPa O_2 compared with 13 kPa O_2 (paired t-test; minute work $P<0.001$; O_2 delivery $P=0.28$; Table 3). Neither power required nor efficacy of O_2 delivery for the highland birds was consistently different from that of the lowland taxa. In 6 kPa O_2, the lowland ruddy duck required a large increase in power to ventilate the lungs, whereas the mallard was estimated to require the largest increase in power output – approximately

threefold. As a result, per unit energy spent, the mallard was estimated to bring much less oxygen to the gas exchange surface than any other bird. The mallard also had the highest cost of breathing, estimated at nearly 16% basal metabolic rate (BMR) in hypoxia. The cost of breathing for all birds was estimated to be 3.5% BMR on average in hypoxia (range 1.52–15.68%), and 0.8% BMR in normoxia (range 0.42–1.04% BMR; see Table 4).

Table 2. Respiratory mechanics measurements made at equal tidal volumes [V_t; as % vital capacity (VC)] and frequencies (f_R)

	Compliance (ml cmH$_2$O^{-1})			τ (s)			Resistance (cmH$_2$O ml^{-1} s^{-1})		
V_t (% VC)	7.3	7.3	14.6	7.3	7.3	7.3	7.3	7.3	14.6
f_R (breaths min^{-1})	20	40	20	20	20	20	20	40	20
Highland									
Puna teal	28.0±0.3	18.7±0.2	21.5±0.3	1.15±0.01	0.90±0.01	1.05±0.01	0.32±0.002	0.27±0.003	0.37±0.004
Speckled teal	39.1±1.2***	19.8±0.7***	25.0±0.9	1.99±0.18*	0.97±0.02	1.29±0.03	0.34±0.02***	0.47±0.01	0.39±0.01***
Ruddy duck	15.1±0.2***	12.0±0.6***	13.7±0.7**	1.54±0.04	0.98±0.07**	1.51±0.11**	0.53±0.01***	0.48±0.03	0.53±0.03
Yellow-billed pintail	25.3±0.7***	14.1±0.3	19.3±0.3***	1.25±0.12*	0.81±0.10	1.08±0.14	0.21±0.01***	0.26±0.02	0.24±0.02
Cinnamon teal	30.1±0.9***	17.6±0.3***	22.1±0.3***	1.29±0.16	0.71±0.05**	1.19±0.08	0.26±0.02***	0.13±0.01***	0.36±0.01***
Lowland									
Green-winged teal	32.3±0.4	24.6±0.1	25.6±0.1	1.53±0.03	1.02±0.01	1.20±0.01	0.51±0.01	0.45±0.002	0.51±0.002
Ruddy duck	23.3±0.5	14.5±0.1	16.9±0.1	1.44±0.04	1.14±0.01	1.18±0.01	0.39±0.01	0.49±0.004	0.46±0.004
Northern pintail	13.7±0.1	12.9±0.7	10.6±0.5	1.14±0.02	0.83±0.03	1.03±0.04	0.27±0.003	0.27±0.01	0.22±0.01
Cinnamon teal	33.9±0.6	19.9±0.8	25.2±1.1	1.55±0.04	0.94±0.01	1.20±0.01	0.41±0.01	0.45±0.01	0.45±0.01
Mallard	11.8±0.3	11.1±0.5	10.8±0.5	1.06±0.03	0.73±0.08	1.10±0.12	0.20±0.005	0.20±0.02	0.17±0.01
Gadwall	15.8±0.3	12.4±0.7	12.7±0.7	1.14±0.04	0.78±0.03	1.19±0.04	0.21±0.006	0.22±0.01	0.20±0.01

The first column for every variable is representative of the average V_t/f_R for all the species at rest (7.3% VC/20 breaths min^{-1}). In the second column f_R is doubled, and in the third column V_t is doubled. Values are means±s.e. Asterisks indicate significant differences between sister taxa (*$P<0.05$; **$P<0.01$; ***$P<0.001$). τ, time constant.

Table 3. Power output and efficacy of breathing at equal tidal volumes and breathing frequencies, and for tidal volumes and breathing frequencies when breathing 13 and 6 kPa O$_2$

	Power (J min^{-1} kg^{-1})			Efficacy (l air J^{-1} kg^{-1})			Power (J min^{-1} kg^{-1})		Efficacy (l O$_2$ J^{-1} kg^{-1})	
V_t (% VC)	7.3	7.3	14.6	7.3	7.3	14.6	Breathing		Breathing	
f_R (breaths min^{-1})	20	40	20	20	40	20	13 kPa O$_2$	6 kPa O$_2$	13 kPa O$_2$	6 kPa O$_2$
Highland										
Puna teal	0.14±0.02	0.44±0.02	0.63±0.14	8.87±0.39	5.64±0.25	4.27±0.15	0.12±0.01	0.24±0.02	1.33±0.11	0.44±0.03
Speckled teal	0.10±0.07**	0.61±0.08***	0.50±0.16	11.9±0.38***	3.82±0.12***	5.04±0.09***	0.18±0.01**	0.50±0.06	1.03±0.08	0.28±0.04**
Ruddy duck	0.17±0.01**	0.56±0.01	0.88±0.02***	2.50±0.23***	1.51±0.14**	1.02±0.10***	0.15±0.01***	0.35±0.01***	0.38±0.03***	0.12±0.002
Yellow-billed pintail	0.13±0.01	0.57±0.01*	0.79±0.03*	5.67±0.17***	2.57±0.08	2.04±0.04	0.14±0.01*	0.36±0.02	0.72±0.03***	0.21±0.01*
Cinnamon teal	0.12±0.01	0.36±0.02	0.33±0.07	8.02±0.42*	5.39±0.28**	6.58±0.26	0.18±0.003***	0.27±0.03***	0.96±0.01	0.39±0.05
Lowland										
Green-winged teal	0.20±0.01	0.26±0.01	0.45±0.02	6.79±0.44	10.7±0.68	6.75±0.40	0.24±0.01	0.37±0.04	0.95±0.04	0.50±0.05
Ruddy duck	0.11±0.01	0.55±0.01	0.67±0.03	7.97±0.73	3.15±0.29	2.76±0.25	0.08±0.01	1.14±0.03	1.24±0.19	0.12±0.003
Northern pintail	0.14±0.01	0.49±0.02	0.62±0.05	2.63±0.15	1.48±0.08	1.39±0.07	0.09±0.01	0.31±0.01	0.37±0.03	0.11±0.003
Cinnamon teal	0.14±0.01	0.35±0.02	0.44±0.11	9.20±0.66	7.27±0.53	6.73±0.44	0.26±0.01	0.65±0.03	0.99±0.03	0.32±0.02
Mallard	0.17±0.01	0.64±0.02	0.95±0.05	2.53±0.21	1.31±0.11	1.03±0.07	0.19±0.02	2.93±0.14	0.30±0.04	0.03±0.001
Gadwall	0.16±0.06	0.65±0.02	1.07±0.07	3.26±0.16	1.61±0.08	1.15±0.05	0.13±0.01	0.41±0.01	0.51±0.03	0.13±0.003

Note that efficacy for equal tidal volumes and breathing frequencies is expressed as efficacy of air delivery, while for *in vivo* values efficacy is of oxygen delivery. Values are mean±s.e. Asterisks indicate significant differences between sister taxa (*$P<0.05$; **$P<0.01$; ***$P<0.001$).

Do birds use an energetically optimal combination of tidal volume and breathing frequency?

As described above, the total work of breathing has two components: the work required to overcome both elastic and resistive forces. When summed, the total work curve for a constant level of minute ventilation tends to be U-shaped, the work required to overcome elastic forces contributing proportionally more at low frequencies and large tidal volumes and the work required to overcome resistive forces contributing more at high frequencies and small tidal volumes. Usually there is an 'optimal' combination of tidal volume and breathing frequency where minute work is minimized. In the present study, the shape of the curves was greatly affected by the extent to which resistive work increased with increasing frequency. In general, the resistive work was small and did not increase much over the range of frequencies and tidal volumes we measured. As a result, most of the curves were not U-shaped.

We plotted the change in power output for the level of minute ventilation used by each species while breathing in normoxia (P_{O_2} of 18 kPa for lowland species and 13 kPa for highland species) and hypoxia (6 kPa P_{O_2}; Fig. 3). We found that when breathing normoxic levels of O$_2$, most birds used a higher V_t/slower f_R pattern than was predicted to be optimal for minimizing the mechanical costs of breathing (Fig. 4). The exceptions were the lowland ruddy duck and the highland speckled teal, which always used the predicted optimal frequency. When oxygen was more limited (P_{O_2} 6 kPa), the highland and lowland cinnamon teals, highland ruddy duck, green-winged teal, northern pintail and mallard moved toward a more optimal combination of V_t/f_R (Figs 3, 4). The puna teal, yellow-billed pintail and gadwall continued to use a slower, deeper breathing pattern than was estimated to be energetically optimal for minimizing the costs of breathing.

DISCUSSION
Morphological and mechanical variation in avian respiratory systems associated with altitude
Our data revealed that birds native to high altitude had increased static respiratory system compliance (Tables 1, 2) primarily due to

Table 4. Estimated cost of breathing for all 11 species of waterfowl

	Normoxia						Hypoxia		
	18 kPa O$_2$			13 kPa O$_2$			6 kPa O$_2$		
Species	BMR (J min^{-1} kg^{-1})	Work of breathing (J min^{-1} kg^{-1})	Cost of breathing (% BMR)	BMR (J min^{-1} kg^{-1})	Work of breathing (J min^{-1} kg^{-1})	Cost of breathing (% BMR)	BMR (J min^{-1} kg^{-1})	Work of breathing (J min^{-1} kg^{-1})	Cost of breathing (% BMR)
Puna teal				361	0.11	0.64	307	0.245	1.60
Speckled teal				373	0.18	0.98	395	0.503	2.56
Ruddy duck				304	0.15	1.02	284	0.346	2.44
Yellow-billed pintail				334	0.14	0.82	336	0.357	2.12
Cinnamon teal				352	0.18	1.04	350	0.267	1.52
Green-winged teal	466	0.23	0.96	534	0.24	0.88	467	0.374	1.60
Lowland ruddy duck	323	0.07	0.42	431	0.08	0.38	525	1.135	4.32
Northern pintail	279	0.07	0.52	346	0.09	0.54	300	0.312	2.08
Lowland cinnamon teal	452	0.20	0.88	531	0.26	0.96	480	0.653	2.72
Mallard	282	0.13	0.90	334	0.19	1.14	373	2.925	15.68
Gadwall	432	0.13	0.58	477	0.13	0.54	414	0.414	2.00

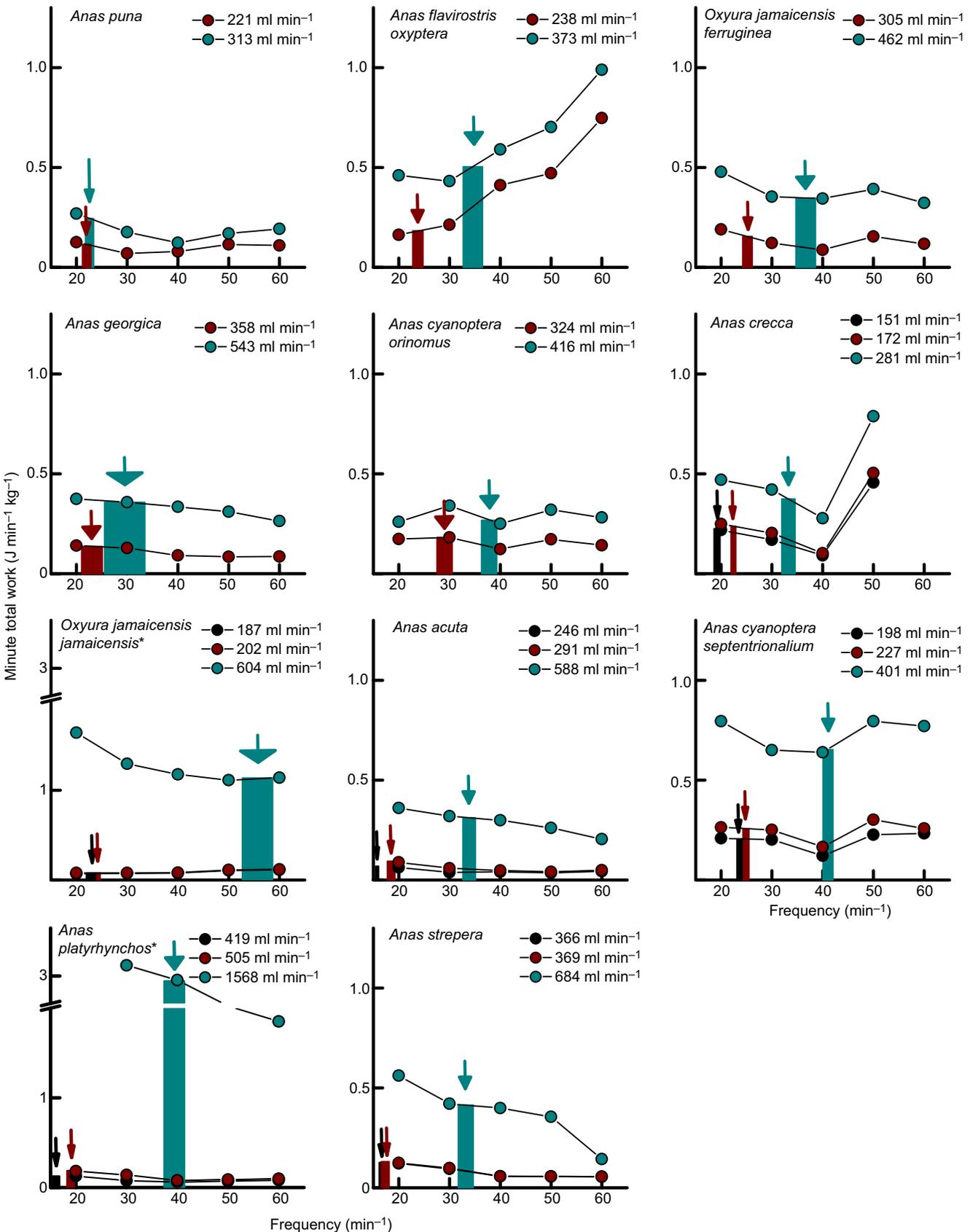BMR (basal metabolic rate) was calculated from the equation given by Romijn and Lokhorst (1966).

Fig. 3. Power output (minute work) at combinations of breathing frequency and tidal volume for minute ventilations measured *in vivo* in 18 kPa O$_2$ (black), 13 kPa O$_2$ (red) and 6 kPa O$_2$ (teal). Lines indicate work for the minute ventilation used by each species at that O$_2$ level (see individual plots for values). Bars with arrows above indicate mean±s.e. of breathing frequencies measured in awake, unanesthetized, resting birds. *Note that the *y*-axis scale is different for the lowland ruddy duck and mallard duck.
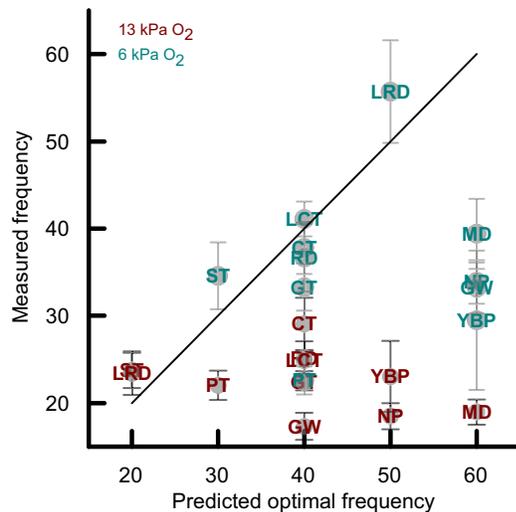
Fig. 4. Measured breathing frequency versus predicted optimal breathing frequency. The optimal frequency is that predicted by the curves in Fig. 3. Black line ($x=y$) indicates where points should fall if birds were using the optimal combination of breathing frequency and tidal volume. Points that fall below this line indicate that birds are expending energy to use a higher tidal volume, points above the line show birds that expend energy to use a higher breathing frequency.

reduced stiffness at low respiratory volumes (removing air below functional residual capacity; Fig. 2). In a mammal that exhales passively, this would have little physiological consequence, but because birds expire actively, even at rest (Powell, 2015), increased expiratory compliance should reduce the elastic work of every breath and allow the highland birds to breathe using larger tidal volumes than lowland birds for a given power output. When the flow component was introduced (dynamic measurements), however, highland species were not found to be consistently more compliant than lowland species and, in the case of the ruddy ducks and cinnamon teals, the highland birds were significantly less compliant. As a result, our estimates of the power output required for breathing was equal or in some cases higher in highland birds at equal tidal volumes and breathing frequencies. It is likely, however, that the increase in static compliance below FRC was not captured by the dynamic mechanics experiments because of the design of the ventilator used in our experiments, which actively inflates but allows only passive deflation, and therefore would not measure any change in work during the exhalation phase. Therefore, it remains possible that the observed reductions in static compliance below FRC could reduce the work of breathing in highland birds, a possibility that awaits further study using different methodology.

Increased respiratory compliance (reduced stiffness) in a bird would be due almost exclusively to changes in the morphology of the body wall, as opposed to in a mammal, whose compliance is primarily determined by the complex lung. In a bird, the thin, avascular air sacs are unlikely to contribute significantly to the overall elastic work; however, this cannot be tested, as any invasive procedure that might isolate the air sacs inevitably punctures them. Air sac compliance would be increased if the overall size of the air sacs is increased, but we saw no evidence of disproportionately larger respiratory systems in the high-altitude taxa. Instead, the increased compliance is likely due to morphological or physiological change in the connective tissue and muscles of the body wall. In birds, the appendicocostalis muscles power inspiration and the external obliques power expiration, and these muscles are

exclusively used for respiration (Codd et al., 2005). An increase in the proportion of oxidative fibers in these muscles might allow them to be thinner and thus contribute to increased compliance, but this would come at the expense of having fewer glycolytic fibers to support non-respiratory high-flow breathing maneuvers involved in airway clearance (e.g. coughing, sneezing; Mantilla and Sieck, 2011).

We found no effect of altitude on extra-pulmonary airway volume, lung mass or heart mass. High-altitude species were significantly larger than low-altitude species following the modified Bergmann's rule previously studied in waterfowl of the Americas (Bulgarella et al., 2007; Wilson et al., 2010), but vital capacity scaled directly with body mass. The lowland mallard and gadwall (not included in statistical tests) had small lungs and large extra-pulmonary airway volumes for their body mass, which may have limited their altitudinal niche and could explain the lack of highland sister taxa for these lineages.

Costs and benefits of deep versus rapid breathing
As predicted, increasing tidal volume was estimated to be a more expensive strategy for increasing ventilation than increasing breathing frequency in most species, with the exceptions of the speckled and cinnamon teals. Increasing tidal volume over breathing frequency reduces the proportion of dead space air, and thus makes ventilation more effective for O_2 uptake. Therefore, the relative benefits of a deeper breathing pattern depend on whether improvements in pulmonary O_2 uptake can outweigh the added O_2 costs of breathing. The greater the dead space volume, the greater the advantage of preferentially increasing tidal volume would be for increasing effective ventilation. Nearly all of the volumes reported here are larger than predicted by the allometric equation of Hinds and Calder (1971), which predicts tracheal dead space volume in birds to be, on average, 4.53-fold larger than mammals of a similar size, so the use of a deeper, slower breathing pattern would likely result in a net benefit for O_2 uptake in these birds despite the added O_2 costs of this breathing strategy. However, this suggestion must be made with some caution because we used extra-pulmonary airway volume as an estimate of dead space volume. The notable exception in this study is the highland ruddy duck, whose estimated dead space volume was 43% smaller than predicted by the allometric equation of Hinds and Calder (1971). This low dead space volume could also reduce buoyancy and facilitate recovery after diving.

The species with the highest respiratory efficacy were the smallest birds, regardless of altitude. This size effect may be related to homeostatic constraints such as the need to maintain higher mass-specific metabolic rates with high heart rates and levels of ventilation at rest. The least efficacious species – the mallard, highland ruddy duck, gadwall and northern pintail – had stiff respiratory systems and either a high airway resistance (highland ruddy duck), or fairly large extra-pulmonary airway volumes (lowland mallard, gadwall and northern pintail).

Work of breathing *in vivo*
Our data indicate that, at rest, the power required to breathe and the efficacy of O_2 delivery was not consistently different based on altitude. However, we note that the least efficacious species were those that have no highland sister taxa – the gadwall and mallard. The mallard in particular was estimated to have brought very little oxygen to the gas exchange surface per unit of energy spent, a finding further supporting the suggestion that respiratory limitations may explain why these lineages have not radiated to altitude.

Cost of breathing at rest

The metabolic cost of breathing for vertebrates at rest has been estimated to be 1–10% of BMR (Otis et al., 1950; Steffensen and Lomholt, 1983; Aaron et al., 1992; Skovgaard et al., 2016; Lee and Milsom, 2016). It has been hypothesized that cost of breathing would be higher in birds because of the heavy weight (on average 17% of body mass) of the pectoral flight muscles attached to the sternum (Markley and Carrier, 2010; Greenewalt, 1962). Previous studies on the cost of breathing in birds have looked at only one species, the helmeted guinea fowl (*Numida meleagris*), and have roughly estimated the cost of breathing at rest to be between 1 and 2% BMR (Ellerby et al., 2005; Markley and Carrier, 2010). In the present study, we were able to estimate the work of breathing without manipulating the breathing strategy. Such manipulations can cause homeostatic imbalance, such as blood pH change that requires active compensation, making metabolic rate estimates unreliable. By temporally separating the measurements of ventilatory and metabolic responses in the awake bird and the work done to achieve those responses in the anesthetized bird, we eliminate this metabolic error. In addition, our work measurements can be applied to any past or future values of breathing frequency and tidal volume that might be measured in these birds. However, converting our 'work' of breathing measurements to 'cost' of breathing requires some assumptions.

The work of breathing estimates were multiplied by 10 to account for respiratory muscle efficiency (10%; Otis et al., 1950) and then doubled to account for the fact that both inspiration and expiration are active. This is the same correction used by Lee and Milsom (2016). We estimated BMR using the equation given by Romijn and Lokhorst (1966):

$$BMR = 16.19 V_{O_2} + 5.00 V_{CO_2}, \qquad (3)$$

where BMR is in kJ, V_{O_2} is the volume of O_2 (l O_2) and V_{CO_2} is the volume of CO_2 (l CO_2). In normoxia, we estimate cost of breathing at rest to be 0.8% BMR in these waterfowl. In hypoxia, this increases to 3.5% BMR on average, but in the case of the mallard was estimated to be as high as 15.7% BMR.

Our experiments were conducted in sternal recumbency (prone position). Sternal recumbency may restrict the rocking movement of the sternum that is used to expand the pleural cavity during normal breathing in birds. However, Malka et al. (2009) found that sternal recumbency was the position that resulted in the greatest air sac and lung volumes measured by CT scan in red-tailed hawks. This was probably due to pleural cavity compression by the flight muscles and intestinal displacement when the birds were placed in dorsal recumbency or on their side (Petnehazy et al., 2012). King and Payne (1964) found a reduction in tidal volume when both conscious and anesthetized chickens were placed in dorsal recumbency as compared with erect posture (sternal recumbency was not measured). Additionally, the *in vivo* respiratory and metabolic values we use here were measured in birds resting in sternal recumbency (C.M.I., S.L.L., J.M.Y., B.A.C., L.A., R.C., N.J.D., P.B.F., K.G.M., W.K.M. and G.R.S., unpublished data). We therefore chose to place the birds in sternal recumbency to measure their respiratory mechanics. This may lead to an underestimation of the work of breathing compared with a standing bird because of a lack of gravitational pull on the mass of the flight muscle attached to the sternum. Tickle and colleagues (2010) noted that barnacle geese sat down more often when carrying a sternal load. They hypothesized that the birds rested on their sternums to change their breathing strategy from sternal rotation to a more energetically favorable costal expansion. In a later study, they found that standing was 25% more metabolically expensive than sitting in sternal recumbency (Tickle et al., 2012). If this is the case, in the present study we primarily measure the work of the costal expansion breathing strategy, and the cost of breathing during standing, running or flight would be expected to be higher.

When do birds breathe optimally?

As already described, there is an 'optimal' combination of tidal volume and breathing frequency where total power output is minimized. We expected that birds would always use this optimal combination. We found that only two species, the speckled teal and lowland ruddy duck, were found to always (in normoxia and hypoxia) use an optimal combination. In normoxia, most species appear to use a slower, deeper breathing strategy rather than minimizing work. In hypoxia, when oxygen was limited, many birds shifted closer to the optimal combination of f_R/V_t. If they did not use the optimum, all species used a slower, deeper strategy, and never a more rapid, shallow strategy than the optimum. This slow, deep breathing pattern should increase effective ventilation and suggests that there could be a trade-off between the increased O_2 cost of breathing versus the increased delivery of O_2 to the lungs, which remains to be determined.

It is important to note that our predictions of the optimal combination of f_R/V_t are somewhat skewed owing to our subtraction of the resistive work created by the intubation tube. *In vivo*, there would be resistance to flow through this portion of the trachea. Typically, the resistive work to move air through the endotracheal tube was approximately 3% of total resistive work, and ranged from 1% at lower frequencies to 10% at higher frequencies. For the low frequencies used by the birds at rest, therefore, the error owing to the subtraction of the endotracheal tube resistive work can be expected to be less than 1%. However, at high frequencies this will reduce the estimated total work of breathing by as much as 10%, and may explain why some of the curves in Fig. 3 are not U-shaped, but continue to decrease with increasing breathing frequency. Given this, optimal combinations may, in reality, be somewhat closer to the deeper, slower combinations used by the birds.

Those ruddy ducks!

The highland ruddy duck did not fit with the high static compliance trend seen in the other highland species. This could be for several reasons. The ruddy ducks were our only diving species in this study, and their respiratory mechanics and morphology may be under different constraints than those in the surface-feeding dabbling ducks. Birds dive upon inhalation, and avian divers may require a stiff chest wall to prevent barotrauma due to excess compression. Anecdotally, the highland ruddy ducks were easy to catch, as they were not able to dive more than once, in contrast with the lowland ruddy ducks, which were very difficult to catch, as they were able to dive repeatedly. This is likely due to an inability of the highland ruddy ducks to replenish O_2 stores rapidly, but this has not been investigated. The lack of consistency may also be due to phylogeny; all other species in this study are in the genus *Anas*, whereas ruddy ducks are distantly related members of the genus *Oxyura*, the stiff-tailed ducks. They may be more closely related to geese than to other duck species (see Fig. 1) (Gonzalez et al., 2009). The non-conforming pattern in respiratory mechanics seen in the ruddy ducks is likely due to a combination of these factors.

We describe these species as being either high or low altitude, and, while some of them are indeed restricted to a specific altitudinal range, birds are highly mobile and using these dichotomous labels

may be somewhat misleading. As previously mentioned, while northern pintails are mainly restricted to low-altitude wetlands in North America (below 2500 m), yellow-billed pintails are found at both high and low altitude in South America, and while the individuals we collected for this study were at high altitude on Lake Titicaca, there is still high gene flow among the population as a whole (McCracken et al., 2009b). The Eurasian subspecies of the American green-winged teal (*Anas crecca crecca*) has been reported to fly over the Himalayas at 5600 m (Groebbels, 1932). Mallards, as another example, are considered a lowland species that never radiated to altitude as they are usually not seen above 2000 m, but there are anecdotal reports of mallards nesting at 3300 m and one report of a mallard (identified by one primary feather) colliding with an airplane at 6400 m (A. Rush, personal communication; Manville, 1963). The close relative of the mallard, the Mexican duck (*Anas diazi*), is also distributed in the highlands in Mexico (Lavretsky et al., 2014). The avian respiratory system is already highly specialized for flight, the most energetically costly form of locomotion, and this may allow birds to move between altitudes with minimal further modification. However, given more evolutionary time, further modification may be required to successfully compete at high altitude.

Conclusions

We set out to address whether there are morphological or mechanical changes in the avian respiratory system associated with life at high altitude and whether any metabolic benefits to these changes reduce the cost of breathing *in vivo*. We found little difference in extra-pulmonary airway volume or lung mass between highland and lowland sister taxa, but estimated that highland species had more compliant respiratory systems below FRC. Despite this, dynamic compliance and resistance were not consistently different, and therefore the work and efficacy of ventilation were similar between highland and lowland taxa. This could reflect exaptation (preadaptation) of the dabbling ducks to high-altitude hypoxia, which would be consistent with the hypothesis that allopatric speciation occurred between these high- and low-altitude taxa because of the lack of wetland habitat at intermediate altitudes (McCracken et al., 2009a). Additionally, the low breathing efficacy of mallards and gadwalls may help explain their inability to expand their altitudinal niche. We estimated the metabolic cost of breathing to be <1% BMR in normoxia and 3.5% BMR in hypoxia, but ranged as high as 16% BMR in hypoxia for the mallards.

We estimated that the ducks were more likely to use an optimal combination of tidal volume and frequency when oxygen was limited. If they did not use the optimal predicted combination, they always breathed slower and deeper rather than more rapidly and shallowly. In theory, this would increase the effective ventilation for a given level of minute ventilation. According to our calculations, for the highland speckled and cinnamon teals, increasing V_t over f_R would also be energetically favorable.

Although we investigated the effect of altitude here, the data presented for the ruddy duck may suggest that waterfowl respiratory mechanics are more constrained by behavior niche (i.e. dabbling, flying or diving) than by altitude. The highly specialized avian respiratory system may lend itself to maintaining oxygen delivery at altitude, so it would be interesting to investigate how altitude affects the respiratory mechanics of mammalian taxa, such as deer mice or bats that inhabit a wide altitudinal range.

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

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