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The role of humidity and metabolic status on lean mass catabolism in migratory Swainson's thrushes (*Catharus ustulatus*)

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Migratory birds use protein as a fuel source during flight, but the mechanisms and benefits of protein catabolism during migration are poorly understood. The tissue-specific turnover rate hypothesis proposes that lean mass loss depends solely on the constitutive rate of protein degradation for a given tissue, and is therefore independent of metabolic rate or environmental stimuli. However, it has been demonstrated that environmental stressors such as humidity affect the rate of lean mass catabolism during flight, a finding that seemingly contradicts the tissue-specific turnover rate hypothesis. In order to resolve this, we placed migratory Swainson's thrushes in either high (HEWL) or low (LEWL) evaporative water loss conditions at rest and while undergoing simulated migratory flight at 8 m s^{-1} in a wind tunnel to test the impact of both environmental stressors and metabolic rate on the rate of protein breakdown. The total quantity and rate of lean mass loss was not different between flight and rest birds, but was affected by humidity condition, with HEWL losing significantly more lean mass. These results show that the rate of protein breakdown in migratory birds is independent of metabolic rate, but it can be augmented in response to environmental stressors.

1. Introduction

Birds are capable of amazing feats of endurance exercise, with some species completing non-stop flights covering up to 11 000 km over 9 days [1]. Endurance exercise in birds is primarily powered by fat metabolism, given the high energy density and storage capacity of fat versus other fuels, and birds have adapted a suite of seasonal physiological adjustments to provide fat at a sufficiently high rate to match the metabolic demands of flight [2–5]. However, there is substantial evidence that birds also catabolize non-fat components before and during migratory flight, even while they maintain adequate fat loads [6–13]. This suggests that a mixture of fat and non-fat components is necessary to sustain endurance flight. However, birds do not seem to rely on glucose metabolism during endurance flight [2,14] owing to the low glycogen loads in birds and the prohibitive weight of such a low-density fuel [15]. Therefore, the non-fat components used during flight are primarily protein. As there is no storage form of protein analogous to triglycerides or glycogen for fatty acids and glucose, respectively, catabolized protein comes directly from functional proteins found in organs such as the digestive tract and muscle during flight [7,8,16]. As a result, migratory birds show dramatic reductions in the sizes of organs after endurance flights [6]. The reductions in size of these organs, particularly digestive organs [17], can result in functional deficits that may influence the stopover requirements, pace of migration, reproductive timing and ultimately the fitness of these animals.

Given the functional costs of catabolizing protein during migratory flight, a suite of hypotheses regarding the benefit of protein metabolism during flight or

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migration have been proposed [6,15,18,19]. For example, protein metabolism in flight results in reduced mass, which lowers the energetic costs of transport, or amino acids may be required to provide substrates required to sustain the high rate of lipid metabolism (for an overview see [15]). One hypothesis that has been gaining support is the protein-for-water hypothesis. During flight, the high ventilation rate required for aerobic metabolism can result in high rates of respiratory water loss [20] and maintaining water balance, particularly for flights over inhospitable and arid environments, has been suggested to be an influential determinant in how far an individual can fly [21–23]. Animals facing high rates of water loss during flight may augment protein breakdown as a means to liberate endogenous water (the sum of water bound to protein and produced from the oxidation of amino acids) [15]. This hypothesis has been tested by flying birds in a wind tunnel under controlled conditions, demonstrating that birds flying under high evaporative water loss (HEWL) conditions catabolize a greater amount of lean mass than when flown under low evaporative water loss (LEWL) conditions, resulting in an increase in the rate of endogenous water production of almost 20% [10].

Resting birds also catabolize significantly more lean mass under water restriction [24], but the rate of protein metabolism was dependent upon the net water balance of the animal. Under resting conditions at room temperature, birds with no access to water catabolized significantly more protein than birds with access to water. However, once moved to a cold trial where respiratory water losses were low, but metabolic rate, and thus metabolic water production, was high, all birds used the same amount of protein. This indicates increased protein catabolism may be a metabolic response to net water loss, rather than being restricted to migratory flight *per se* [24]. Although resting birds have much lower ventilation rates than flying birds [20], they too have lower rates of endogenous water production from fat and protein catabolism. If the protein-for-water hypothesis is the primary driver of lean mass catabolism, then we predict that the amount of lean mass lost may be dependent upon the net water balance (water loss through respiration and water gain through endogenous sources). We would then expect that lean mass dynamics in migratory birds will be related to metabolic rate and environmental conditions that a bird experiences.

Another hypothesis that has been gaining support is the tissue-specific turnover rate hypothesis. The rate of tissue turnover is driven by rates of protein breakdown and protein synthesis [19,25,26]. This hypothesis proposes that protein breakdown is constant and independent of energy expenditure, while protein synthesis is determined by nutritional status (i.e. fasted versus fed) [26]. Many migratory bird species undergo fasting incidentally while flying as they do not feed on the wing, so protein synthesis may be suppressed without a corresponding suppression in protein degradation leading to net protein loss. It has been suggested that this process is responsible for determining the differential reduction in organ mass that has been observed in migratory birds after long duration flight [6]. For example, organs that have the highest rate of turnover, such as liver and intestine routinely show the most dramatic reductions in mass after long duration flight or fast compared to other tissues such as the heart [26]. However, it is currently unknown how acute activity level influences the lean mass dynamics of an animal, as the basis of this hypothesis is from a study on chronically challenged birds over a 10 week period [19]. If this hypothesis is true, then a fasted bird at rest will have the same amount of lean mass loss as an actively migrating bird.

These two hypotheses may not be mutually exclusive and do not exclude the possibility that other stressors can result in accelerated protein degradation rates that may be mechanistically separate processes from one another. The current body of experimental evidence in birds undergoing a simulated migratory flight support the protein-for-water hypothesis, but no study has yet examined how multiple proposed mechanisms may relate. For instance, we do not know if the protein-for-water hypothesis and the tissue-specific turnover rate hypothesis are both supported under experimental conditions and are capable of interacting with protein degradation pathways. One possibility is that protein breakdown is, in part, driven by both environmental variability and is independent of metabolic rate.

Here, we investigate the tissue-specific protein turnover hypothesis and protein-for-water hypothesis in simulated migration to better understand how environmental stressors and tissue-level turnover rates determine the overall rates of protein breakdown in migratory birds. To achieve this goal, we used quantitative magnetic resonance (QMR) body composition analysis and long duration flights in a climatically controlled wind tunnel to measure changes in body composition of Swainson's thrushes (*Catharus ustulatus*), a long distance Nearctic-Neotropical migrant, when exposed to high and low evaporative water loss conditions during long duration flight and also at rest. If lean mass catabolism is augmented owing to the metabolic requirements of flight, then there should be different rates of lean mass loss between flight and rest birds. If lean mass catabolism occurs simply as a product of protein turnover, then there should be no difference in the rates of protein loss between the two metabolic states. Furthermore, if protein catabolism is in response to environmental stressors such as humidity, then there should be higher rates of protein loss in birds exposed to HEWL compared to LEWL conditions.

2. Methods

(a) Animals

Twenty-nine Swainson's thrushes were captured at Long Point Bird Observatory (Long Point, ON, Canada) during their southbound migration between 6 and 9 September 2017, banded with a unique colour band combination for identification, and transported to the Advanced Facility for Avian Research at Western University, London, ON, Canada. Birds were held on a 12 L : 12 D photoperiod at 20°C and approximately 60% relative humidity in 2.3 × 2.4 × 3.5 m indoor free-flight aviaries. A synthetic diet (electronic supplementary material, table S1) and water was provided ad libitum, and approximately 10 *Tenebrio* mealworms per bird were provided daily. Birds were held for at least two weeks before beginning experimental procedures.

(b) Flight protocol

Birds were flown in a temperature, pressure, and humidity controlled wind tunnel at the Advanced Facility for Avian Research [27]. The wind tunnel is capable of maintaining relative humidity from 0% to 90% to upwards of 30°C (for a description of the properties of the wind tunnel, see [10,28]). Birds were flown at 8 m s^{-1} at 18°C, in either 12 g H_2O m^{-3} (LEWL) or 2 g H_2O m^{-3}

(HEWL) humidity, which correspond to 80% and 12% relative humidity, respectively, similar to Gerson & Guglielmo [10]. The dry condition corresponds to relative humidity and temperatures passerine species may experience while migrating over the Sahara or other arid landscapes [29]. All flights were matched with one rest bird that underwent the same handling, but simply sat in a covered cage within the wind tunnel plenum for the duration of the flight for the flight bird. Because many Nearctic-Neotropical migrants (including Swainson's thrushes) migrate at night, all flights began 30 min after lights off, which corresponded to 19:30 Eastern daylight time zone. Three hours before the flight, up to four birds (one rest and three candidate fliers) were collected from the free-flight aviary and placed in cages (61 cm × 50 cm × 50 cm) with access to only fresh water. Immediately before flight, the rest bird and the first candidate flight bird were weighed (to the nearest 0.001 g), and scanned using QMR technology to determine fat mass, lean body mass, and total body water [30]. The rest bird was placed into a covered cage within the plenum of the wind tunnel, and the flight bird was released into the air stream. We aimed for each bird to achieve steady state flight for a minimum of 30 min. If a bird was unable to achieve steady state flight during this time, the bird was removed from the wind tunnel and replaced with another candidate flier that was weighed and QMR scanned immediately prior to its release into the air stream. As a result of potentially flying up to three different birds in a single evening, some rest birds spent more time within the wind tunnel than their paired flight bird. The first condition a bird experienced (rest versus flight, HEWL versus LEWL) was randomly selected.

Once a bird achieved steady flight, we followed a 'three strikes' rule to determine maximum voluntary flight duration [31], where a flight was ended if a bird stopped flying three times within 5 min. Birds were allowed to fly up to a maximum flight duration of 12 h. However, some flights were terminated before a bird reached its maximum voluntary flight duration to achieve a range of flight durations. Upon completion of the flight, the bird was weighed, QMR scanned, and blood sampled via brachial puncture using a 24-gauge needle into heparinized capillary tubes (Fisher Scientific, Pittsburgh, PA). Blood sampling occurred within 5 min of the conclusion of the flight. Plasma was separated by centrifugation at 2000*g* for 10 min and frozen at −80°C.

Of the 29 birds brought into captivity, only 11 birds demonstrated willingness to fly in the wind tunnel. Select birds were flown multiple times to increase the total number of successful flights (total number of flights = 19; HEWL = 10, LEWL = 9; total number of rest trials = 18; HEWL = 9, LEWL = 9). Once recovered, flight birds were also used as rest birds for subsequent flights. All birds were allowed a minimum of 3 days to recover between experimental flights or resting bouts. Every effort was made to ensure that birds underwent all factorial conditions (rest versus flight, HEWL versus LEWL) within the migratory season of the Swainson's thrush. However, some birds were not able to be placed in all conditions owing to time constraints. Five additional birds were selected to be included as rest birds. One flight did not have a rest bird. Three flight birds did not undergo a rest condition.

(c) Plasma metabolite analysis

All plasma was thawed on ice and diluted threefold in 0.9% w/v saline solution. All assays were performed on a fluorometric/spectrophotometric 96 well plate reader (BioTek Synergy H1, Winooski, VT, USA). Uric acid was measured fluorometrically using the Amplex Red Uric Acid/Uricase Assay Kit (A22181, ThermoFisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions. Glucose was measured using the Amplex Red Glucose/Glucose Oxidase Assay Kit (A22189, ThermoFisher Scientific, Waltham, MA, USA).

(d) Calculations and statistics

Changes in body composition over the course of a flight, as measured using QMR, were used to calculate flight energy expenditure. The energy content of fat and lean mass was assumed to be 39.6 kJ g wet mass^{-1} and 5.3 kJ g wet mass^{-1}, respectively [15], and the summed energy content was divided by duration in the wind tunnel (either flying or at rest) to calculate metabolic rate. The production of metabolic water was calculated from fat and lean mass loss, assuming 1.10 g of H_2O g wet fat mass^{-1} and 0.82 g H_2O g wet lean mass^{-1}, respectively [15]. Per cent body water was determined by dividing total body water by wet lean mass. Lean body mass was used instead of whole-body mass to calculate the per cent body water because of the large amount of body fat these birds attained before entering the wind tunnel, and the low amount of water contained within adipose tissue [32] produced unrealistically low per cent body water. The change in per cent body water was calculated as the difference between per cent body water before and after entering the wind tunnel.

All statistical analyses were performed in R v.3.4.4 [33]. Because our study is an unbalanced repeated measures design, linear mixed effects models were used throughout, using package lme4 (v1.1-17 [34]). Mixed-effects models were used to take into account unbalanced repeated measured on individuals because of the missing values for some of these individuals [35]. Two different random effects were compared using Akaike information criterion scores corrected for small sample sizes (AICc) before starting backwards model selection of the fixed effects (described below): (i) including only bird identity (ID) as a random intercept only and (ii) including bird ID and flight duration as a random intercept-random slope. However, the best fitting random effects for all models only included bird ID.

The initial fixed effects included a three-way interaction between flight condition (rest versus flight), humidity condition (low versus high evaporative water loss), and duration in the wind tunnel (in hours), and the initial fat mass before entry into the wind tunnel. Backwards model selection was used to determine the fixed effects for each dependent variable, using AICc to determine the best fitting model [36]. If the AICc score of two competing models differed by less than 2, the model with the fewest predictors was selected [37]. The final model was refitted using restricted maximum-likelihood estimates to get parameter estimates of each variable in the final model. We examined the change in whole-body mass, change in fat mass, change in wet lean mass, change in total body water, proportion of energy derived from lean sources, proportion of water derived from lean sources, and change in per cent body water in this manner. For the analysis of metabolic rate and metabolic water production, we did not include initial fat mass in the full, starting model.

Body mass, fat mass, wet lean mass, total body water, and per cent body water of each trial were compared among groups using mixed-effects models as above. However, we only included tarsus length of a measure of body size and a two-way interaction of humidity condition and flight condition before commencing model selection.

Residuals of the final model were inspected for normality. Because proportions are bound by 0 and 1, the proportion of water and energy derived from lean sources were logit transformed before undergoing mixed effects linear modelling analysis [38]. Data is presented as mean ± standard error. Final models can be viewed in the electronic supplementary material, table S2.

3. Results

Birds did not vary in initial body mass (35.536 ± 0.883 g; all effects: $p > 0.05$), initial fat mass (8.495 ± 0.895 g; all effects:

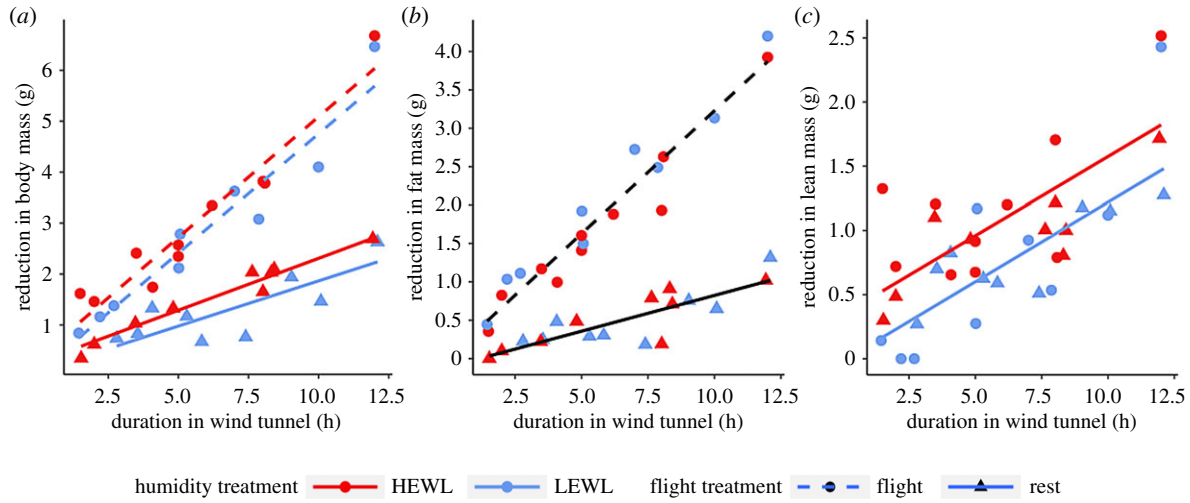

Figure 1. The effect of duration in the wind tunnel on the reduction in (*a*) whole-animal, (*b*) fat, and (*c*) lean mass losses under HEWL (red) and LEWL (blue) conditions. Regression lines are of the best model, with black indicating if relative humidity was a not significant factor, or red and blue (for HEWL and LEWL, respectively) if relative humidity was retained. Triangles and circles are rest and flight birds, respectively. (Online version in colour.)

$p > 0.05$), or lean mass (23.355 ± 0.391 g; all effects: $p > 0.05$) after accounting for structural size using the tarsus measurement. However, birds in the LEWL condition had 0.846 ± 0.308 g higher total body water than HEWL birds ($t = 2.782$, $p = 0.0103$). Despite this difference in total body water, there was no difference in per cent body water between groups (85.2 ± 0.04%; all effects: $p > 0.05$). Flight duration in the wind tunnel ranged from 87–720 min for both HEWL and LEWL flights (HEWL: mean 5.87 h, range: 1.50–12 h; LEWL: mean: 6.30 h, range: 1.45–12 h).

(a) Mass loss and duration in the wind tunnel

Flight birds lost significantly more body mass compared to rest birds at equivalent flight lengths, as indicated by the interaction between the metabolic state (flight versus rest) and duration in the wind tunnel (flight treatment: $t = 0.235$, $p = 0.816$; duration: $t = 16.991$, $p < 0.001$; flight treatment × duration interaction: $t = -7.573$, $p < 0.001$; figure 1*a*). Flight birds lost mass at a rate approximately 2.9 times higher than rest birds (model slopes of 0.161 ± 0.028 g h^{-1} in rest and 0.459 ± 0.027 g h^{-1} in flight). Furthermore, humidity had a significant effect upon body mass loss, with HEWL birds having a 0.372 ± 0.108 g greater loss of body mass than LEWL ($t = -3.444$, $p = 0.002$; figure 1*a*). As a result, the *y*-intercept of the HEWL flight birds was 0.470 ± 0.190 g ($t = 2.470$, $p = 0.019$) and the *y*-intercept of the HEWL rest birds was 0.533 ± 0.212 g ($t = 2.517$, $p = 0.017$). The *y*-intercept of LEWL rest and LEWL flight birds was not significantly different from zero ($p > 0.05$). However, there was no significant interaction between humidity and duration in the wind tunnel or metabolic state, so the effect of humidity was only additive.

The reduction in fat was only affected by the duration in the wind tunnel and the metabolic state of the animal, with a significant interaction between duration and metabolic state (flight treatment: $t = -0.520$, $p = 0.607$; duration: $t = 18.208$, $p < 0.001$; flight treatment × duration interaction: $t = -9.154$, $p < 0.001$; figure 1*b*). The slope of the relationship between duration in the wind tunnel and fat mass loss in rest birds was 0.087 ± 0.018 g fat h^{-1}, compared to the flight birds, which had a slope of 0.321 ± 0.018 g fat h^{-1}. There was no effect of humidity upon the reduction in fat mass. Of note, one short flight under HEWL conditions did not consume any fat mass, according to QMR measurements. The *y*-intercept was not significantly different from zero ($p > 0.05$).

The reduction of lean mass was significantly higher in HEWL than LEWL groups, with HEWL birds losing 0.360 ± 0.122 g more lean mass than in LEWL at an equivalent flight length ($t = -2.938$, $p = 0.006$; figure 1*c*). Metabolic state did not have a significant effect upon lean mass loss. Duration in the wind tunnel significantly affected lean mass loss, with a slope of 0.122 ± 0.019 g of lean mass h^{-1} ($t = 6.327$, $p < 0.001$; figure 1*c*). The *y*-intercept of the best fitting model is significantly different from zero under HEWL conditions, having a value of 0.351 ± 0.143 g ($t = 2.458$, $p = 0.02$). For LEWL conditions, the *y*-intercept was not significantly different from zero (−0.009 ± 0.150 g; $t = -0.059$, $p = 0.953$).

Apparent total body water declined with time spent in the wind tunnel at around 0.095 ± 0.028 g h^{-1} ($t = 3.38$, $p = 0.002$). This apparent rate only takes into account initial and final total body water measurements, and is not indicative of the rate of water turnover in the animal. Metabolic state, initial fat load, and humidity treatment did not have an effect on total body water loss. There was no change in per cent body water between the beginning and end of the experimental treatments ($p > 0.05$).

(b) Water production and metabolic energy sources

The mean rate of energy expenditure was only influenced by metabolic state, with flight costs of 3.73 ± 0.12 W and rest costs of 0.96 ± 0.16 W or about 3.86 times greater metabolic rate in flight compared to rest ($t = -17.70$, $p < 0.001$; figure 2*a*). HEWL and LEWL treatments did not have a significant effect on metabolic rates for either metabolic state.

The proportion of overall energy generated from lean mass was higher in rest birds than in flight birds (flight treatment: $t = 6.219$, $p < 0.001$; figure 2*b*), owing to the relatively low amount of fat catabolized compared to flight birds. However, the effect of metabolic state was only additive. Humidity treatment had a significant effect on the proportion of energy from lean sources, with LEWL treatment groups having a lower contribution of lean mass to the total energy expenditure than the HEWL group ($t = -4.410$, $p < 0.001$). Furthermore, longer durations in the wind tunnel were

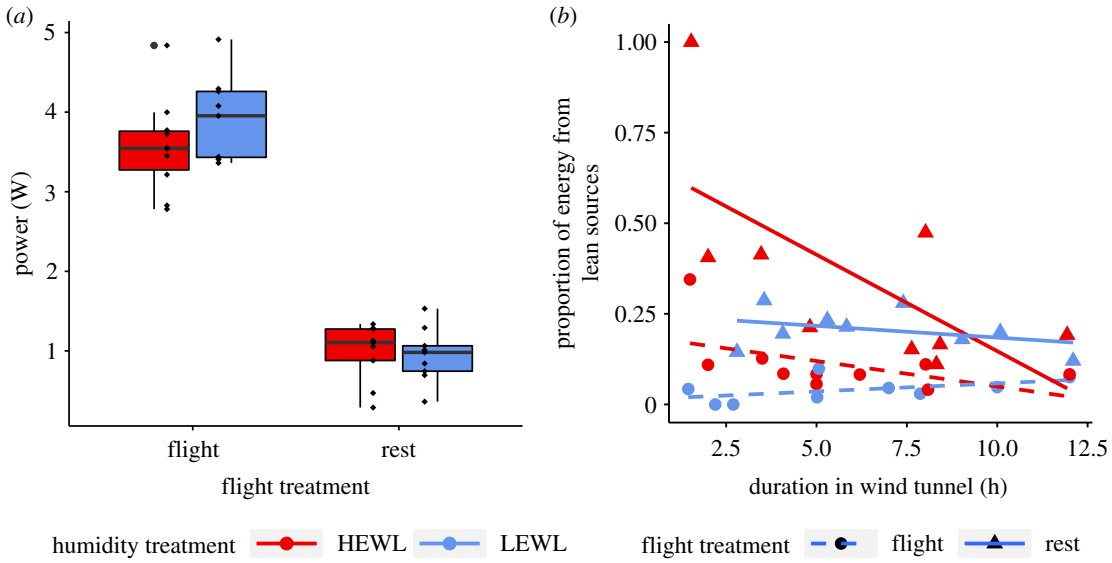

Figure 2. (*a*) The whole-animal metabolic rates of rest and flight birds under HEWL (red) or LEWL (blue). (*b*) The relationship between time in the wind tunnel with the proportion of lean mass to total energy expenditure under HEWL (red) and LEWL (blue) conditions. Triangles and circles are rest and flight birds, respectively. Individual data points overlay (*a*) as diamonds. (Online version in colour.)

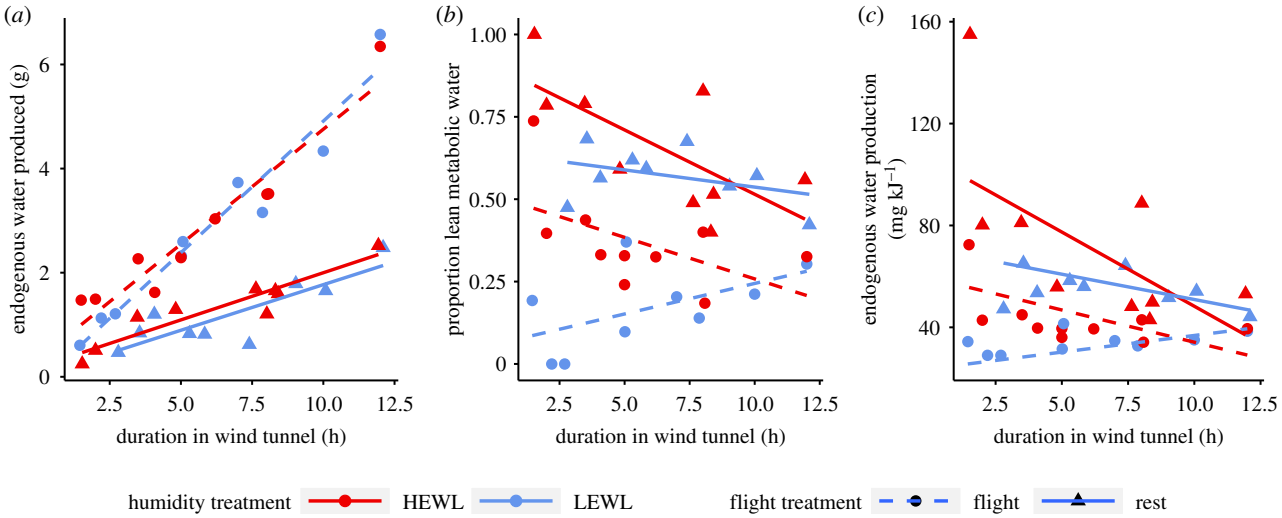

Figure 3. The relationship between duration in the wind tunnel and (*a*) total endogenous water production, (*b*) proportion of water from lean sources, and (*c*) endogenous water production per unit energy, under HEWL (red) and LEWL (blue) conditions. Triangles and circle are rest and flight birds, respectively. (Online version in colour.)

associated with a lower contribution from lean sources than shorter durations ($t = -2.980$, $p = 0.005$). There was a significant interaction between duration in the wind tunnel and humidity treatment ($t = 3.174$, $p = 0.004$), with HEWL birds having a steeper decline in proportion of energy from lean sources with time in the wind tunnel compared to LEWL birds. There was a highly influential data point from a single rest bird. Reanalysis after the removal of this point showed the same overall pattern, but different parameter estimates (see the electronic supplementary material 2 for parameter estimates of the alternative model).

Only duration in the wind tunnel and flight treatment had an effect on the amount of total endogenous water produced over the course of the flight, with more time spent in the wind tunnel leading to greater amounts of endogenous water produced (flight treatment: $t = 0.189$, $p = 0.851$; duration: $t = 16.641$, $p < 0.001$; figure 3*a*). Further, there was a significant interaction between duration in the wind tunnel and flight treatment (duration × flight treatment interaction: $t = -7.481$, $p < 0.001$), resulting in rest birds producing water at around 0.163 g h^{-1}, compared to 0.470 g h^{-1} in flight birds.

A greater proportion of the water was derived from lean in rest birds than flight birds ($t = 7.109$, $p < 0.001$; figure 3*b*). HEWL treated birds also derived a greater proportion of their total water from lean mass than LEWL birds ($t = -4.858$, $p < 0.001$). The contribution of lean mass to water declined with longer durations in the wind tunnel ($t = -3.290$, $p = 0.002$). There was a significant interaction between humidity treatment and duration in the wind tunnel ($t = 3.521$, $p = 0.001$), suggesting that this decline was more pronounced in the HEWL group than the LEWL group.

Rest birds had a lower endogenous water production per unit energy than flight birds ($t = 5.453$, $p < 0.001$; figure 3*c*). Furthermore, HEWL had higher energy specific water production rates than LEWL, but owing to the interaction between humidity treatment and duration in the wind tunnel, declined with longer flights (humidity treatment:

$t = 3.520$, $p = 0.001$; duration: $t = -3.875$, $p < 0.001$; humidity treatment × duration interaction: $t = 2.647$, $p = 0.012$). LEWL energy specific water production rates were relatively constant for the entire duration in the wind tunnel.

(c) Plasma metabolites

Blood glucose was not significantly different among flight treatments, humidity treatment, or durations, with a mean glucose concentration of 18.85 ± 3.88 mM across all flight and rest birds. The only significant factor for plasma uric acid was flight treatment ($t = -2.247$, $p = 0.0324$), with flight birds having a higher plasma uric acid of 0.70 ± 0.38 mM, while rest birds had nearly half the amount of uric acid (0.43 ± 0.29 mM). Humidity and flight duration did not have a significant effect on plasma uric acid.

4. Discussion

(a) Lean mass catabolism and flight

This is, to our knowledge, the first study to compare the acute effects of metabolic state (rest versus exercise) on lean mass dynamics in birds in a controlled experimental setting. Rest and flight birds catabolized lean mass at the same rate, suggesting that metabolic rate is not an important determinant of the rate of protein loss. Instead, ambient humidity determines the relative proportion of lean mass to the overall fuel mixture. Bauchinger *et al*. [19] has previously demonstrated that protein turnover rates are not influenced by metabolic rate, suggesting that lean mass loss during flight should not be different than at rest, assuming the environment is the same. However, Bauchinger *et al*. [19] used a chronic metabolic challenge to study carbon turnover and organ reduction to generate the tissue-specific turnover rate hypothesis, so the role of metabolic rate upon organ mass reduction during migration has not, to our knowledge, been examined until this study.

Here, we show that instead of metabolic rate, ambient humidity was a key regulator of lean mass catabolism. In addition to humidity manipulation in flying Swainson's thrushes [10], research has found evidence to support the protein-for-water hypothesis in other water-restricted animals, such as house sparrows (*Passer domesticus*) [24], gravid Children's pythons (*Antaresia childreni*) [39], and Richardson's ground squirrels (*Urocitellus richardsonii*) [40,41]. Based on the diverse array of species that use protein as a source of endogenous water, augmentation of the rate of protein catabolism in response to environmental stimuli may be a broadly employed strategy across taxa in response to water stress. However, the proportion of water and energy derived from protein catabolism is probably species and context specific, with environmental conditions, season, life history, nitrogen biochemistry, and renal physiology playing key roles in determining both the role and the degree of protein breakdown. For example, Rutkowska *et al*. [42] has found that zebra finches (*Taeniopygia guttata*), a species that is native to arid regions of Australia, are capable of relying upon fat metabolism in the face of dehydration stress. A large multispecies comparison across environments will provide greater insight into the role of proteins during dehydration stress, and how differing life and evolutionary histories may determine the strategies that species use. Further, the molecular mechanisms that regulate lean mass catabolism during net water loss are not well understood. One possibility for the high lean mass loss under water restriction or dehydration stress may be a glucocorticoid-mediated response. Studies on birds captured mid-migration have demonstrated that leaner birds tend to have higher circulating corticosterone and higher plasma uric acid than fatter birds, with the high uric acid suggesting increased protein catabolism [43]. Overall, this suggests that there may be a role for glucocorticoids in regulating protein metabolism.

Flight duration has a profound effect upon the rate of lean mass loss, with high lean mass loss occurring early in a flight and declining as flight progresses. This decline in the rate of lean mass loss is indicated by the non-zero intercepts in the linear models examining whole-animal and lean mass loss against duration. Because a non-zero intercept suggests that birds immediately lose mass upon entry into the wind tunnel (i.e. there is a significant change in mass at time = 0), which is extremely unlikely, we can infer that the relationship between lean and whole-body mass loss and duration is non-linear. Most likely, the rates are initially very high for the first 90 min, and then rates decline as duration increases. However, because we do not have durations below 90 min, we are unable to say with certainty how the rate of mass loss changes during the initial phases of flight.

The relationship between the rate of lean mass catabolism and flight duration has been noted in other bird species. In yellow-rumped warblers (*Setophaga coronata*), lean mass contributes a greater proportion of energy early in flight and then declines as the flight duration increases [31]. Birds shift in fuel mixture as flight progresses, with pigeons transitioning from a primarily carbohydrate mixture to primarily lipid metabolism within 30 min [44]. However, the glycogen content of bird tissues is relatively low compared to mammals [45], and maintaining a store of carbohydrates may be important for burst flight for predator escape and to supply organs that preferentially catabolize glucose as their primary fuel. Once lipid metabolism has achieved a high enough flux to support the metabolic demands of flight, lean mass catabolism declines as flight progresses. This reduction may be driven by the inhibitory effect of ketone bodies upon gluconeogenesis and amino acid metabolism of glucogenic amino acids [46]. With the rise of fat oxidation during the early portions of flight, plasma ketone bodies concurrently increase [11,31,47,48], which in turn will inhibit the rate of lean mass loss through gluconeogenesis. The inhibition of gluconeogenesis by ketone bodies may explain the decline in lean mass loss rates with increasing duration in the wind tunnel.

There is a marked difference in the proportion of overall metabolic rate and endogenous water production that is provided by lean mass, with flight birds having a much lower reliance upon lean mass than their rest counterparts. As these are migratory birds during their autumn migration, sparing of adipose stores may be an important strategy during resting and overnight periods, and other fuels such as protein and intracellularly stored triglycerides may be preferred [49]. There is evidence that shorebirds will reduce the size of organs (e.g. digestive organs) before departing from their staging grounds [13], one potential reason being to reduce flight costs by lowering the amount of metabolically active tissue mass or to power metabolism to prevent lipid oxidation. Swainson's thrushes in this study may be relying upon something similar, with increased protein catabolism and organ mass reduction during overnight resting periods in

order to spare fat. Furthermore, reductions in lean mass may also provide additional energetic savings through reduced mass of metabolically active tissues, and preserve the metabolically inactive adipose mass [50]. Overall, reductions in lean mass, particularly within tissues not needed for flight such as digestion, may spare fat from oxidation during rest. It should be noted that protein catabolism is difficult to capture through non-invasive methods, and this may be the first direct evidence of fat sparing in a small passerine species at rest. Respirometry measurements of birds in migratory phases of their life history have a respiratory quotient (RQ) of around 0.7, suggesting that fat or protein is the primary fuel used to power metabolism [51]. However, because uricotelic animals have an RQ of around 0.74 when catabolizing proteins, which is nearly identical to the RQ of fat, it is difficult to discern fat or protein catabolism in a resting migratory birds through respirometry [52]. Further investigation into this possibility could rely on isotopically labelled diets and isotopic breath analysis to more precisely discern fuel sources [53].

The lack of variation in overall metabolic water production, despite the higher consumption of lean mass in HEWL conditions, seems to indicate that fat catabolism contributes a sizable amount of the water and energy during flight, particularly in the later stages of flight when protein catabolic rates begin to decline. This is in contrast to the rest birds, which derived a greater proportion of their water from protein. The high rate of fat use may reduce the amount of water that needs to be generated from other non-fat sources (such as protein). Other studies have suggested that water produced though the metabolism of fat alone may be sufficient to offset any water loss experienced during water restriction in a non-migratory bird species, such as the zebra finch [42], but it is important to note the possibility of differences between desert adapted birds, such as the zebra finch, and migratory birds in their metabolic response to environmental stressors. In order to offset the higher loss of water in the HEWL environment, birds needed to additionally catabolize a greater amount of lean mass relative to the HEWL environment. However, the greater contribution of lean mass catabolism did not significantly alter the overall energy density of the fuel mixture, owing to the low energetic density of protein compared to fat [15]. Interestingly, there is no difference in plasma uric acid levels between HEWL and LEWL groups, despite the increase in lean mass catabolism occurring in the HEWL condition. Given that there is no evidence of reduced kidney function during flight compared to birds at rest [28], uric acid may continue to be filtered and excreted during flight.

We did note a slight difference in initial water mass across the humidity treatments. This initial difference may be driven by initial lean mass, as the HEWL individuals were slightly smaller, even though this was not significant. However, we did not see any change in per cent body water with flight treatment, humidity treatment, or time spent in the wind tunnel despite seeing a decline in overall total body water, with birds maintaining a constant per cent body water through the duration of the experimental treatments. This suggests that the relative amount of water contained in their tissues is defended, and makes up a constant proportion of lean body size. Overall, birds do display a tendency to maintain approximately 70% body water (relative to total body mass) within their tissues [7,42], and any deviation from this probably compromises physiological function.

The flight metabolic rates measured here using QMR are slightly lower than previously reported metabolic rates in wind tunnels (3.7 W reported here versus 4.2 W in [10]), but close to metabolic rates of wild Swainson's thrushes during migration [54]. This difference in flight metabolic rate to other wind tunnel studies may stem from the different ages in the cohorts, with primarily hatch year birds used here compared to second year birds in the previous study [10]. The difference in moult between hatch year and second year birds may allow for lower costs of flight. However, the most likely effect in this study is the lower preferred flight speed ($8\ m\ s^{-1}$) compared to prior studies where $10\ m\ s^{-1}$ was used [10]. The power curve relating the energy cost to flight speed and age has not yet been determined for Swainson's thrushes, however. The resting metabolic rates using QMR technology measured here is approximately two times higher than previously reported basal metabolic rates [55], assuming an RQ of 0.7. The high resting metabolic rates are probably owing to the low temperature, which is outside the thermal-neutral zone of Swainson's thrushes, and birds were not always calm during the resting trials in the wind tunnel, particularly when the birds were first placed in the cage.

Behaviour plays an important role in determining flight success in wind tunnel studies and can be affected by many factors, including weather cues [56]. We sought to avoid flight training, which would confound our analyses. Because all birds were captured mid-migration over a relatively short timeframe, the lack of cooperation to fly in the wind tunnel by some individuals is probably not owing to physiological or population-level differences. Furthermore, Swainson's thrush are long-distance migrants [57], and do not have any resident populations that remain in Canada during the autumn migration, ensuring all birds used in this study were physiologically capable of long duration flight.

In summary, metabolic rate does not influence the rate of lean mass catabolism in migratory birds. Instead, the amount of lean mass lost is, in part, dependent upon the sum total of the tissue-specific turnover rate of each individual tissue [26]. Environment also plays an important role in modulating the rate of lean mass catabolism, with dryer environments eliciting greater protein breakdown than more humid environments. These findings support two of the leading hypotheses concerning lean mass catabolism, and suggest they are not mutually exclusive but, rather, complementary.

Ethics. All procedures were approved by Western University Animal Care Committee (Protocol 2010-216) and the University of Massachusetts Amherst Institutional Animal Care and Usage Committee (2015-0019). Birds were collected with permission from the Canadian Wildlife Service (permit CA0256, issued to Dr Christopher Guglielmo).

Data accessibility. All data are available in the electronic supplementary material.

Authors' contributions. D.J.E.G. and A.R.G. conceived of the study. D.J.E.G., J.E.D., M.C.L. and A.R.G. collected the data. D.J.E.G. carried out the statistical analyses and drafted the manuscript. A.R.G., J.E.D. and M.C.L. helped revise the manuscript. All authors gave final approval for publication.

Competing interest. We declare we have no competing interests.

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