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Long-duration wind tunnel flights reveal exponential declines in protein catabolism over time in short- and long-distance migratory warblers

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During migration, long-distance migratory songbirds may fly nonstop for days, whereas shorter-distance migrants complete flights of 6 to 10 h. Fat is the primary fuel source, but protein is also assumed to provide a low, consistent amount of energy for flight. However, little is known about how the use of these fuel sources differs among bird species and in response to flight duration. Current models predict that birds can fly until fat stores are exhausted, with little consideration of protein's limits on flight range or duration. We captured two related migratory species—ultra long-distance blackpoll warblers (*Setophaga striata*) and short-distance yellow-rumped warblers (*Setophaga coronata*)—during fall migration and flew them in a wind tunnel to examine differences in energy expenditure, overall fuel use, and fuel mixture. We measured fat and fat-free body mass before and after flight using quantitative magnetic resonance and calculated energy expenditure from body composition changes and doubly labeled water. Three blackpolls flew voluntarily for up to 28 h—the longest wind tunnel flight to date—and ended flights with substantial fat reserves but concave flight muscle, indicating that protein loss, rather than fat, may actually limit flight duration. Interestingly, while blackpolls had significantly lower mass-specific metabolic power in flight than that of yellow-rumped warblers and fuel use was remarkably similar in both species, with consistent fat use but exceptionally high rates of protein loss at the start of flight that declined exponentially over time. This suggests that protein may be a critical, dynamic, and often overlooked fuel for long-distance migratory birds.

migration | blackpoll warbler | yellow-rumped warbler | flight energetics | physiology

Migratory birds cover extraordinary distances to seasonally exploit favorable habitats and conditions in distant locations. In songbirds (Passeriformes), these journeys are accomplished in a series of nonstop flapping flights operating at roughly 8 to 10 times their basal metabolic rate (BMR) alongside increased rates of water loss (1–3). These journeys can range from short- to long-distances, even within closely related species, with long-distance migrants crossing vast ecological barriers over thousands of kilometers in nonstop flights lasting for days, while short-distance migrants complete overland routes in a series of overnight flights with ample landing opportunities (3–6). Migratory birds seasonally alter their capacity to transport and catabolize fat to fuel their demanding flights, which provides the vast majority the fuel needed to make the journey (2, 7, 8). Indeed, fat is the most energy-dense fuel available to the bird and it is stored efficiently in lightweight adipose tissue with minimal bound water (9).

Though fat is the primary fuel source for migratory flight, protein is estimated to contribute 4 to 7% of the total flight energy (8, 10, 11). While the role of protein catabolism in migratory flight is not fully understood, it was long assumed that protein was spared as a fuel of last resort because it was documented as reductions to vital organs and muscles following nonstop flight (12–16). Therefore, the estimated ~5% of flight energy coming from protein may lead to functional losses that can impose important limits on flight duration and animal performance after flight (17).

Our current understanding of fat and protein use and aerodynamics is frequently used to estimate chemical and mechanical power as well as migration distance and duration (12, 18, 19). *Flight v. 1.24* (20) is one steady-flight modeling program that incorporates the physical principles of flight alongside assumptions of fuel contributions and muscle efficiency, and it has been applied to estimates of flight cost and migration distance for multiple species of birds (21–23) and bats (24). The bird model applies the assumption that protein catabolism consistently contributes 5% of flight energy, with the remaining 95% coming from fat (10, 23, 25). The "Migrate" function of the program therefore performs a time-marching calculation of changes to body mass—and accompanying

Significance

Birds primarily burn fat during migratory flight, and the current assumption is that protein is burned consistently at low levels, incidental to fat. We compared fat and protein use in wind tunnel flights between closely related short- and long-distance migratory birds and found remarkable similarity, with consistent fat use but high rates of protein breakdown early in flight that decline over time. Migratory blackpoll warblers flew nonstop for up to an unprecedented 28 h in the wind tunnel, voluntarily finishing with ample fat but reduced flight muscle size. These long-duration trials allow the most comprehensive picture of fuel use in migratory flight to date.

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alterations to drag, power coefficients, wingbeat frequency, and other covariates—until the fat stores of the simulated bird are exhausted (20, 26). These models often result in extremely low final fat-free mass, but the role of protein in determining flight range has not been experimentally examined. However, records of birds arriving at their destinations with emaciated muscles but remaining fat stores (27) suggest that real-world flight range may be constrained by protein loss.

Protein may also be a more responsive and beneficial fuel than previously acknowledged. For example, migratory white-throated sparrows (*Zonotrichia albicollis*) show a seasonal increase in the capacity to catabolize protein (28) and migratory songbirds flown in a wind tunnel show greater lean mass loss in response to drier conditions, indicating that lean mass may serve as a source for endogenous water during flight (17, 29, 30). Protein catabolism in migratory flight may also restore Krebs cycle intermediates that enable continued fatty acid catabolism (31), maintain optimal power to weight ratios as fuel loads change in flight (32), or reflect an imbalance between consistent, tissue-specific rates of protein catabolism and suppressed rates of protein synthesis (33). If protein catabolism serves an important function in migratory flight independent of fat catabolism, it may show variability that exceeds our current view of fuel use in migratory flight.

One way to examine the importance of protein catabolism in flight is to compare flight fuel use in related migratory species that differ greatly in their migratory strategies. Many studies generalize migration physiology to all migratory species, but the extent to which flight physiology differs between short- and long-distance migrants is unclear. These migratory strategies lead to selective pressures of different types and severity, leading to differences in enzyme activity (34), endurance capacity (35), and muscle physiology (36, 37), but they may also differ in flight performance, efficiency, water balance, and fuel use. If protein catabolism serves a vital function for migratory flight, such as alleviating water stress, we might expect to see canalization of this trait for a more prominent humidity response in long-distance migrants during flight relative to short-distance migrants. Alternatively, if protein catabolism is simply a by-product of flight metabolism, such as a source of Krebs cycle intermediates for fat catabolism, we may expect to see protein breakdown correlates closely with the degree of fat catabolism, and the degree of this relationship may be determined by migratory distance.

We tested the underlying assumption that endurance flights are fueled by consistent rates of fat and protein catabolism in migratory birds. To accomplish this, we compared the fuel use strategies of related ultra-long and short-distance migratory *Setophaga* warblers in captive wind tunnel flights under dry or humid conditions. While blackpoll warblers have been documented flying nonstop for over 100 h across open water during their migratory flights (38), yellow-rumped warblers complete shorter 6 to 10 h hops overnight and primarily over land. This provides an ideal comparison for drastically different migratory strategies. Before and after flights, we weighed the birds and measured body composition using quantitative magnetic resonance (QMR) (39) and compared calculations of flight metabolic power using QMR as well as doubly labeled water (DLW). We predicted that energy expenditure, overall fuel use, and fuel mixture in response to the humidity conditions would differ between the short- and long-distance migrants. If protein is adaptive for migratory flight and a humidity response, we might expect to see a more prominent protein loss response to dry conditions in long-distance migrants. Alternatively, if protein loss is detrimental, we may expect to see a lower overall reliance on protein as a fuel source in long-distance than short-distance migrants (11, 36).

Methods

Capture and Housing. In fall 2018, 20 blackpoll warblers *Setophaga striata* (Forster 1772) and 44 yellow-rumped warblers *Setophaga coronata* (Linnaeus 1766) were captured using mist-nets at Long Point Bird Observatory in Long Point, ON, Canada. Blackpoll warblers were captured between 12 and 27 September and yellow-rumped warblers were captured between September 12 and October 13. Blackpoll warblers are ultra-distance neotropical migratory songbirds that depart their boreal forest breeding range in North America in the fall and gather on the Atlantic coast before flying nonstop for ~100 h over open water to their winter range in northern South America (4, 38). Meanwhile, yellow-rumped warblers migrate shorter distances primarily over land to the southern United States, Caribbean Islands, and Central America with nocturnal flights that likely last 6 to 10 h (3).

Birds were color banded for identification and transported to the Advanced Facility for Avian Research at Western University in London, ON, Canada, where they were housed in free-flight rooms (2.4 m × 3.6 m) or paired in cages (121 × 68 × 185 cm) under a 12 h light/12 h dark photoperiod aligning with normal daylight hours and approximately 20 °C and 60% relativehumidity (RH). Birds were maintained on an ad libitum synthetic high-carbohydrate diet modified from Guglielmo et al. (40) with supplemental *Tenebrio* mealworms provided daily (41). Blackpoll warblers were on average 12.43 g upon capture (range 10.54 to 14.65 g) with muscle scores of mostly 2 on the 0 to 3 scale outlined by Redfern et al. (42) and fat scores ranging from 0 to 4 (mean = 1.7) on the 0 to 8 scale according to Kaiser (43). Yellow-rumped warblers were captured with fat scores ranging from 0 to 3 (mean = 1.4) and muscle scores from 2 to 3 (mean = 2.1), with total body mass ranging from 10.24 to 13.50 g (mean = 11.72 g). We began monitoring the condition of captive birds during the experiment using QMR body composition analysis (39) on October 1 for blackpoll warblers and on November 1 for yellow-rumped warblers. Blackpoll warblers all began storing significant amounts of fat by the second week of monitoring, increasing from an average fat mass of 2.98 g (range 1.55 to 6.31 g) to 8.10 g (range 2.60 to 12.52 g; *SI Appendix*, Fig. S1). For the duration of the experiment, Yellow-rumped warblers maintained stable body composition, with an average mass of 13.25 g (range 11.26 to 15.42 g) and fat mass of 2.32 g (range 0.50 to 4.12 g). Procedures were approved by the University of Massachusetts Amherst Institutional Animal Care and Use Committee (2015-0019) and the Western University Animal Use Subcommittee (2010-216) and collected under Canadian Wildlife Service permit #CA0256 issued to Dr. Christopher Guglielmo.

Experimental Design. All birds were given at least 2 wk to acclimate to captivity prior to flight experiments in the wind tunnel. We monitored yellow-rumped warblers overnight with infrared cameras to confirm migratory restlessness in their cages. For a description of the wind tunnel, see Gerson and Guglielmo (29). To determine whether the environmental conditions influence fuel use, we flew birds overnight in minimal light conditions under both high evaporative water loss (HEWL; 2 g H2O m^{-3}; 13% RH) and low evaporative water loss (LEWL; 12 g H2O m^{-3}; 80% RH) conditions at 18 °C at 8 m/s wind speed. This wind speed was previously selected for yellow-rumped warblers to encourage flight in the wind tunnel and has been used for numerous experiments (17, 40, 44, 45). For comparable measurements between the two warbler species, we also flew blackpoll warblers at the same speed. However, we acknowledge that a limitation of the wind tunnel is our inability to adjust wind speed to match ideal flight speeds as fuel loads decrease for each individual (23, 26). Given that the U-shaped flight power curve is nearly horizontal near the minimum power speed (V_{mp}), as was recently seen in blackcaps flown in a wind tunnel (45), a bird flying slightly slower or faster than V_{mp} will have a similar chemical power output, and even migrating birds crossing major barriers may not be selecting their maximal range speed (V_{mr}) during flight (46). We estimated the power curve for both species across flight speeds using the *afpt* (1.1.0.2) package in R (47) to demonstrate that 8 m/s falls near the estimated V_{mp} for all birds that were flown in the wind tunnel (*SI Appendix*, Fig. S2). Furthermore, these estimates and the wind speed selected for the wind tunnel are unlikely to be an accurate approximation of absolute air speed recorded in the wild. For example, if we estimate the flight speed of blackpoll warblers tracked during fall migration over the Atlantic in the study by Deluca et al. (4), the average absolute ground speed of approximately 13 m/s (range 7.9 m/s to 19.6 m/s) clearly exceeds the V_{mr} estimated for our birds and demonstrates the difficulty in extrapolating speeds recorded in wild birds to flights in captivity.

Flights began approximately 30 min after lights were turned off in the evening as both species are nocturnal migrants. Access to food was restricted for 1 h prior to flight. Additional flight protocol details can be found in the study by Gerson et al. (17). Eighteen blackpoll warblers were selected for experiments, with nine flying in the wind tunnel at the facility and others serving as rest birds that were concurrently held in a covered cage in the plenum of the wind tunnel room to experience the same conditions alongside the flight. For yellow-rumped warblers, 29 birds were included in the experiment and 16 birds were flown. Before and after flights, we weighed the birds and measured fat mass using QMR. We regularly test the accuracy of fat mass measurements in the QMR by scanning known oil standards as in ref. 39. Although fat-free mass constitutes all nonfat body components including the beak, feathers, bones, and carbohydrates, the bulk of the mass that is changing during flight comes from lean mass changes due to protein catabolism.

Initially, flights were capped at 6 h and birds were flown under HEWL and LEWL for repeat measures. Flights were ended if the bird attempted to land three times within a 5 min period, leading to numerous flights under 6 h. Despite capping flights, some measurement durations exceeded 6 h when the rest bird remained in its cage while the flight bird was captured, weighed, and scanned using QMR. Birds flew up to four times each, and we eliminated 5 rest and 3 flight blackpoll warblers from the analysis to account for instances where the QMR showed abnormal variability in the oil standard readings or bird scans, such as those leading to an estimated gain in fat or fat-free mass after the flight or rest. We ended the experiments with 12 flight and 8 rest measurements for blackpoll warblers in HEWL conditions and 9 flight and 5 rest measurements for LEWL conditions. For yellow-rumped warblers, we ended with 15 flight and 12 rest measurements in HEWL and 14 flight and 14 rest for LEWL conditions. Each of these was solo flights. We also allowed three blackpoll warblers to begin uncapped flights together in LEWL conditions until each bird voluntarily stopped, at which point we removed that bird from the tunnel for measurement, while the remaining birds continued flying. We measured total body mass changes with a balance (0.001 g), while fat was measured immediately before and after flight using QMR. Fat-free mass was used as our proxy for wet lean mass by subtracting dry fat mass from total body mass.

Energy Expenditure. Flight costs are typically measured using mask respirometry, ^{13}C-labeled sodium bicarbonate (NaBi), DLW, or QMR (45, 48). QMR has been used repeatedly for wind tunnel flights, and QMR-measured changes in fat and fat-free mass between scans are used to estimate energy expenditure by multiplying these changes by the energy density of the tissue (37.6 kJ/g for fat mass or 5.3 kJ/g for wet lean mass). However, this method has not been directly compared to measurements from traditional methods such as DLW.

We calculated energy expenditure from DLW using breath isotope enrichment measurements (49) using an LGR Water Vapor Isotope Analyzer (Los Gatos Research, San Jose, CA) and standard calculations from (50). Initially, we collected background breath isotope enrichment on each bird to apply a correction to enriched breath. This was only measured once for each bird after they were in captivity for over 2 wk due to the consistent background enrichment measurements over time. After lights out, we injected birds intraperitoneally with approximately 19 mg (range: 10.9 to 26.9 mg) prepared 2:1 ^{18}O:^{2}H DLW (Cambridge Isotope Laboratories, Tewksbury, MA). The syringe was weighed to the nearest 0.001 g before and after injection to determine the dose. One hour after injection, we measured the breath enrichment of the birds using a face mask setup until the isotope measurements plateaued (equilibration measurement). After the bird flew or rested, we collected breath enrichment again. The logarithm of the turnover in these enrichment values for ^{18}O and ^{2}H over the time between measurements was used to calculate the elimination constants for each isotope during flight per hour (k_o and k_d, respectively). Because QMR total body water measurements were inconsistent, we compared pre- and post-flight body water pools using 66% of the fat-free mass and took the average of these for the estimated flight body water pool (N). We calculated rCO_2 (mol/h) using the one-pool model equation 7.17 in Speakman (50), simplified to $rCO_2 = N((0.48123 \times k_o) - (0.48743 \times k_d))$, which we then converted to Watts.

Flight Simulations. We used the Migrate function in *Flight 1.24* (20) to simulate flights for blackpoll warblers. Aside from the defaults, we incorporated parameters for blackpoll warblers used by Bayly et al. (22), including wing span (0.2108 m), wing area (0.0080 m^3), and flight muscle fraction of 0.156, which come from closely related Bay-breasted warblers (*Setophaga castanea*) and blackburnian warblers (*Setophaga fusca*) as reported in the study by Graber and Graber (51). We also used a BMR factor of 1.2 as this was the closest approximation of the preflight resting metabolic rate of yellow-rumped warblers from a previous wind tunnel study (17) and the fat-free mass-corrected estimate of the metabolic rate measured in our rest birds using QMR and DLW. The altitude of the simulations was set to 0 m above sea level to be comparable to the wind tunnel. The speed control was set using the V/V_{mp} ratio (flight speed vs. minimum power speed), and when possible we adjusted this ratio to match 8 m/s constant flight speed. For nine of the birds with higher preflight masses, it was not possible to set this ratio below 1, so the simulated speed was higher than 8 m/s (maximum speed was 9.07 m/s). Therefore, for these birds, we acknowledge that the range estimate would be higher than expected if the bird was flown at 8 m/s due to the higher flight cost at lower speeds (see *Discussion* of wind tunnel limitations above). We ran the Migrate program with these parameters and adjusting the total mass and fat fraction for all of the blackpoll warblers that flew in the wind tunnel. For each time step in the simulated migratory flights, we calculated fat-free mass by subtracting the fat mass from total mass, and then calculated the cumulative fat and fat-free mass lost since the starting point and the rate of fat and fat-free mass loss over the elapsed time.

Statistical Analysis. Data are available in supplemental material (Dataset S1). All statistics were performed in R software (v 4.1.1, R Foundation for Statistical Computing, Vienna, Austria). We used the function "lmer" (lme4 package, version 1.1-17) and performed backward fixed-effects stepwise model selection on linear mixed-effects models with a $\alpha = 0.05$ cutoff for fixed effects. Bird ID was included as a random effect to account for repeated measures for each individual bird. For starting models, we were interested in how the rate of mass loss (fat or fat free) relates to the starting body mass, flight or humidity treatment, species, duration in the wind tunnel, and the interaction of duration × species or duration × flight treatment.

All energy expenditure statistics were conducted using the mass-specific metabolic power (Watts/gram) due to the significant difference in starting masses for yellow-rumped warblers and blackpoll warblers ($t = 3.34$, df = 16.6, $P = 0.004$). Our starting model examined the difference in mass-specific metabolic power with fixed effects for the two measurement techniques and their interaction with the flight status, the humidity treatment, species, and duration in the wind tunnel, with bird ID as a random effect due to repeated measures.

Results

Body Composition. Fat was burned consistently as a fuel for both species, with a higher rate of fat loss in flight (0.15 ± 0.01 g/h) than at rest (0.06 ± 0.01 g/h; $F_{1,88.99} = 52.79$, $P < 0.001$) (Fig. 1). A similar pattern appeared with the rate of fat-free mass loss ($F_{1,72.23} = 14.49$, $P < 0.001$), but the rate of fat-free mass loss also declined exponentially with flight duration ($F_{1,66.60} = 47.64$, $P < 0.001$) (Fig. 1). Three blackpoll warblers flew until they voluntarily stopped, leading to flights of approximately 10, 20, and 28 h, respectively. After these long-duration flights, substantial fat reserves remained (>3 g), but concave flight muscle and fat-free mass of ~8.5 g indicate that flight was limited by lean body mass rather than fat loss. As in the shorter flights, fat was burned consistently higher in flight (0.14 ± 0.01 g/h) than rest (0.04 ± 0.01 g/h; $F_{1,42} = 118.41$, $P < 0.001$), and the rate of fat-free mass loss was slightly lower in rest birds than that in flight birds ($F_{1,40.70} = 6.60$, $P = 0.014$) and declined exponentially with flight duration ($F_{1,24.14} = 76.23$, $P < 0.001$) to the point where fat-free mass contributions dropped from approximately 10% to less than 3% of total energy (Fig. 2).

Energy Expenditure. Estimated metabolic power for blackpoll warblers in flight was 1.46 ± 0.2 W using QMR and 1.62 ± 0.2 W using DLW, and for yellow-rumped warblers, it was 1.43 ± 0.2 W with QMR and 1.50 ± 0.1 W with DLW (Fig. 3). For both species,

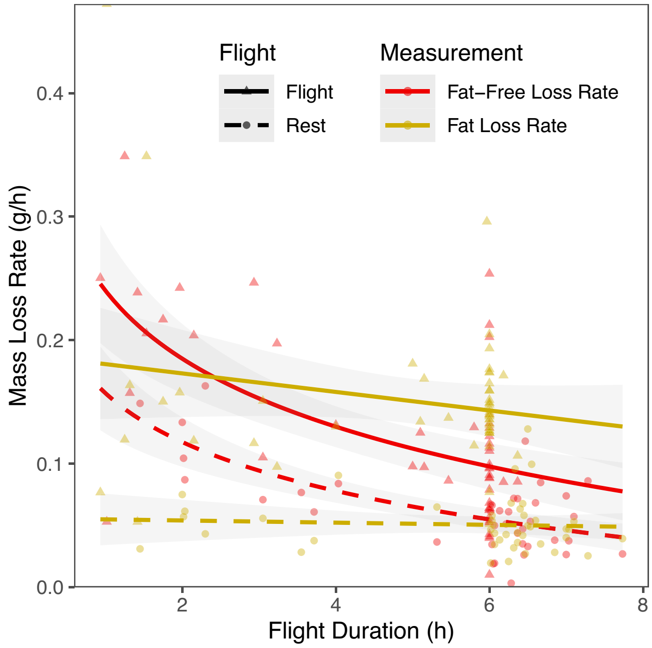

Fig. 1. Fat mass is lost consistently over flight or rest, while the rate of fat-free mass loss is high initially and declines exponentially over time in both yellow-rumped warblers and blackpoll warblers. The two species are merged in this figure since there was no significant difference in fuel use for these comparable flights lasting up to ~6 h. The rate of fat mass loss is shown in yellow and fat-free mass loss is shown in red, with flight birds shown with solid lines/triangles and birds at rest depicted in dashed lines/circles. N = 50 flight and 39 rest measurements.

this was approximately 2.9 times the metabolic rate of the resting birds (0.49 ± 0.1 W). When using mass-specific metabolic power, we found a significant interaction between the measurement technique (DLW or QMR) and the flight status (flight or rest) ($F_{1,51.1}$ = 9.64, P = 0.003). Therefore, we split the model by flight status and continued with backwards model selection. For flight birds, we found a significantly greater mass-specific metabolic power in yellow-rumped warblers ($F_{1,11.8}$ = 11.76, P = 0.005) with a trend toward 0.005 ± 0.003 W/g lower estimates using QMR than that of DLW ($F_{1,28.3}$ = 4.12, P = 0.052). For rest birds, the final model showed that QMR estimates were approximately 0.005 ± 0.002 W/g higher than that of DLW ($F_{1,20.0}$ = 7.21, P = 0.014) and mass-specific metabolic power was higher for the HEWL treatment ($F_{1,32.8}$ = 4.28, P = 0.047).

Flight Simulations. We ran *Flight 1.24* Migrate simulations using the preflight total masses and fat fractions for all blackpoll warblers flown in our experiment (*SI Appendix*, Table S1). These simulations predicted that the birds in our study would fly between 27 and 164 h (average of 82 h) at approximately 8 m/s for a final distance of approximately 2,431 km (range 769 to 5260 km), ending with no fat remaining and body masses ranging from 6.6 to 13.7 g (average of 9.6 g). This was a more severe ending body composition than we saw in the blackpoll warblers that were allowed to fly to a voluntary end point, with shorter flight durations overall in the wind tunnel and the lowest fat-free mass at the end being approximately 9.6 g and a minimum of 3.3 g of fat remaining.

Discussion

Despite vast differences in the distances and barriers traversed by short- and long-distance migrants, we found remarkably similar fuel use for yellow-rumped warblers and blackpoll warblers flown in a wind tunnel. Though yellow-rumped warblers showed higher mass-specific metabolic power, fat was used consistently as a fuel. Importantly, both warblers initially burned protein at a higher rate than expected—making up over 10% of flight energy—followed by a dramatic reduction over the first several hours of flight. This pattern was most apparent with the addition of three voluntary long-duration blackpoll warbler wind tunnel flights lasting up to 28 h—the longest wind tunnel flight to our knowledge. These flights ended with ample fat stores but depleted flight muscles. These wind tunnel flights in closely related *Setophaga* warblers shed light on the dynamic use of protein in migratory flight.

Despite the difference in their migratory distance, there were surprisingly few differences between yellow-rumped warbler and blackpoll warbler fuel use in wind tunnel flights up to 6 h. Fat was a consistent fuel, with more burned in flight than in rest, but the rate of fat-free mass loss was highest early in flight. This rate declined exponentially with flight duration, eventually settling around 2.5% of flight energy in the longest flights for blackpoll warblers. A similar pattern appeared in birds at rest, but to a lower degree than the corresponding flight birds. High fat-free mass loss early in flight may point to protein as an important fuel initially during migration. In American Robins (*Turdus migratorius*), a gradual reduction in lean mass catabolism within the first hour of flight in a wind tunnel was shown previously by Gerson & Guglielmo (52), but we provide evidence that this reduction continues over several hours of flight. These temporarily high rates of protein catabolism contradict the assumption that protein contributes consistently to flight fuel over each iterative step of a simulation (23). This could alter predictions of flight ranges, for example, if vital weight adjustment early in flight results in lighter loads that ultimately save fuel for the remainder of the journey.

Rapid protein loss in the first hours of flight provides insight into the use of protein to fuel migratory flight. This may indicate digestive organ catabolism early in flight to reduce the weight and

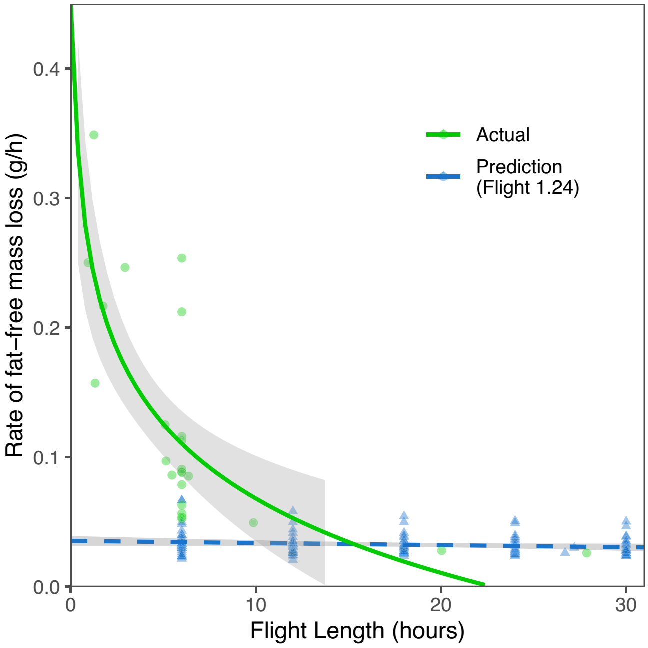

Fig. 2. The rate of fat-free mass loss in wind tunnel flights for all blackpoll warbler flights is exceptionally high initially and declines exponentially over time in flight, in contrast to the predictions of consistent protein loss using *Flight 1.24*. Simulations (blue/triangles/dotted line) incorporated the starting total and fat masses of all blackpoll warblers flown in the wind tunnel in the Migrate program, with 5% of flight energy coming from protein and flight speed set to 8 m/s. Corresponding measurements for rates of fat-free mass loss in blackpoll warbler flights up to 28 h are shown in green/circles/solid line. N = 24 flights from 9 different birds.

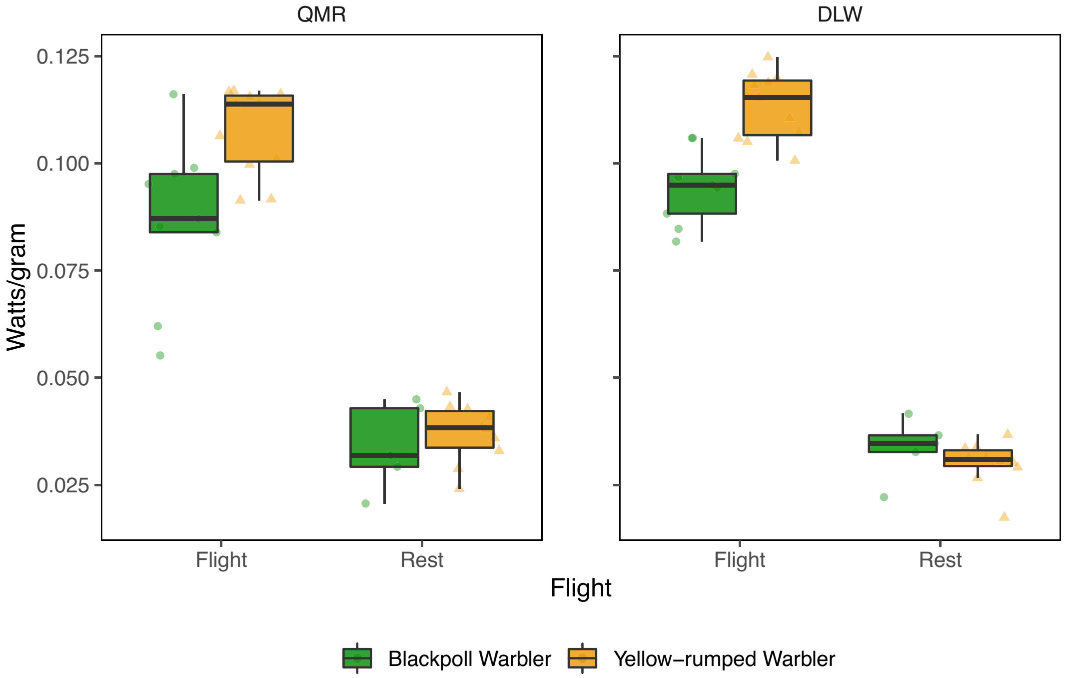

Fig. 3. Mass-specific metabolic power is significantly lower in blackpoll warblers than that in yellow-rumped warblers in flight, but not in rest. Data are subset for flights in which quantitative magnetic resonance (QMR) and doubly labeled water (DLW) energy expenditure measurements were both completed for flights up to ~6 h. In general, QMR measurements (*Left*) of metabolic rate were higher in rest birds and lower in flight birds than those of DLW measurements (*Right*). Blackpoll warblers are shown in green/circles and yellow-rumped warblers in orange/triangles. N = 9 flight and 7 rest for blackpoll warblers and N = 12 flight and 10 rest for yellow-rumped warblers.

energy expenditure of an active tissue that does not contribute to flight, as was seen prior to migratory flight in bar-tailed godwits in ref. 53, though this adjustment has not been shown to continue into flight before. However, Bayly et al. (27) found that blackpoll warblers caught after arriving on the coast of Colombia after their long-duration flight did not appear to have reduced digestive capacity upon arrival. These blackpoll warblers may not show the same degree of digestive organ catabolism shown previously in shorebirds, although gut enzymes may compensate for reduced gut mass to maintain digestive function (54). Furthermore, if this were simply a way to save weight in flight, it is unlikely that we would see a similar pattern in the resting (and fasting) birds unless it is a predictive adjustment when birds are in migratory condition as seen in ref. 53, which could be tested by measuring rates of protein catabolism in the same species in nonmigratory condition. This would be particularly interesting if rapid protein breakdown at the start of flight or fasting provides an influx of uric acid as an antioxidant in the migratory condition (55, 56). By showing a reduction in the protein contribution to flight fuel over time, we also show that the protein loss, at least initially in flight, is unlikely to be solely an iterative adjustment to fuel loads throughout flight (16, 26). As fat was burned consistently, we would have expected correlated, gradual fat-free mass loss to adjust to these changing fuel loads. The difference in the degree of fat-free mass loss between flight and rest birds does provide some support for the hypothesis that protein serves to replenish Krebs cycle intermediates during fatty acid metabolism. However, if this were the sole purpose of the protein catabolism, we might also expect correlated rates of both fat and fat-free mass loss over the duration of flight or rest to replenish depleted intermediates, or perhaps even an increase in protein catabolism over time as the pool is depleted below a threshold (57), although previous wind tunnel studies suggest that the switch to fat catabolism occurs within the first hour of flight (8) and the initial protein catabolism may have a different purpose than what is seen later in flight or may be a result of higher corticosterone (CORT) levels early in flight (58). Finally, the protein turnover hypothesis suggests that protein catabolism occurs at consistent, organ-specific rates regardless of the exercise status and must be balanced by protein anabolism, which is typically suppressed during flight or fasting. In this hypothesis, the different degree of fat-free mass loss seen between flight and rest in this study may be explained by different degrees of suppression of protein synthesis, while the exponential reduction in fat-free mass loss rates over the duration of flight or rest may reflect degradation of organs with high rates of protein turnover followed by those with lower rates while synthesis is suppressed (59). While our findings shed light on these hypotheses individually, they are not mutually exclusive and none are entirely refuted by these data. For example, we can only speculate on the timing of organ-specific contributions to lean mass loss in flight that may give rise to the changes seen in the study by Schwilch et al. (60), so there may be more dynamic catabolism of one organ system, such as the gut, while the flight muscle is reduced more consistently alongside fuel loads.

Interestingly, we did not see a significant effect of the humidity treatment on the fat or fat-free mass loss in either species. While there was a trend toward higher fat-free mass loss in the HEWL flight birds, this was not significant. Although this response has been consistent and robust in many species of migratory birds, including yellow-rumped warblers in previous studies (17, 29, 30), long-distance migratory birds like blackpoll warblers may have an attenuated response to spare protein during extended flight durations (36). Additionally, protein breakdown early in flight may be related to a CORT stress response (61), which may be greatest prior to flight or early in flight (58). The use of DLW in this study required an additional degree of handling over the hour prior to starting the flight compared to previous studies' flying yellow-rumped warblers, which could have increased the preflight CORT response more uniformly for all individuals. The period of substantial fat-free mass loss we found early in flight may also be the time period where a humidity effect leads to the greatest differences if it is a CORT-mediated response to maintain water balance (62), and by handling the birds more prior to flight we may have dampened the degree of this difference. However, it is unlikely that the pattern of fat-free mass loss seen was fully the result of handling-induced CORT as we found no significant difference in the amount of fat-free mass loss between birds that did or did not receive the DLW treatment ($F_{1,39.5} = 0.187$, $P = 0.67$), and studies have shown that CORT responses to acute stress return to baseline levels within 30 to 60 min (63).

Despite finding no significant differences in fuel use for the two species, we did find lower mass-corrected metabolic power for blackpoll warblers than yellow-rumped warblers. Metabolic power for blackpoll warblers has not been measured directly in a wind tunnel flight before, but the *afpt* power curve estimate of approximately 1.67 ± 0.3 W at 8 m/s was close to our measurement using DLW (1.62 ± 0.2 W) and about 14% higher than our QMR estimate (1.46 ± 0.2 W). For blackpoll warblers, this means flight metabolic costs are roughly 6.3 times the predicted BMR of the birds as predicted by the allometry equation from McKechnie and Wolf (64) of roughly 0.22 W, though no experimentally determined BMR is available for blackpoll warblers for comparison. For yellow-rumped warblers, our measurement was comparable to published flight costs using the NaBi method (1.5 ± 0.4 W), though our QMR estimate was 9 to 40% lower than previous measurements for the species using QMR (40, 45). Their flight costs represent roughly 7.3 times the predicted BMR of 0.19 W (64), though previous studies using migratory yellow-rumped warblers have shown a higher resting metabolic rate than predicted [0.256 ± 0.038 W in the study by Gerson et al. (17)] that would correspond with a 5.6 times increase in metabolic rate during flight. The higher mass-specific flight metabolic power in yellow-rumped warblers than blackpoll warblers, measured by both QMR and DLW, may reflect selective pressures for efficient flight in ultra-long-distance migratory species crossing major ecological barriers (25, 36, 65) where poor condition or inefficient flight can lead to high mortality (66, 67). In contrast, yellow-rumped warblers typically remain over land and even those with poor flight performance would likely survive the migratory journey through a series of shorter-duration hops. Hahn et al. (35) speculated that physiological differences such as muscle fiber sizes and vasculature (37) or mitochondrial efficiency (68) could lead to differences in exercise endurance seen between short- and long-distance migrants. Since flight energy expenditure differences appeared using both QMR and DLW measurements, it is surprising that no differences in fuel use appeared. However, slight, nonsignificant differences in fuel use, such as the minor trend we see toward a higher rate of fat loss in yellow-rumped warblers, could compound over long flight durations and lead to this variation in flight energy expenditure. Furthermore, as these differences appeared in the mass-specific metabolic power in flight only, it likely reflects more efficient flight behavior or aerodynamics in the blackpoll warblers that allows them to transport a greater fuel load for roughly the same total fuel cost as the yellow-rumped warblers. While we did not conduct wing measurements in our individual birds, blackpoll warblers show some of the highest wing aspect measurements of *Setophaga* warblers (69), and these more pointed wings in long-distance migrants can significantly reduce flight costs (70).

While calculations of energy expenditure using QMR and DLW supported the higher mass-specific metabolic power in yellow-rumped warblers and the correlation between the two measurements was strong, QMR measurements were slightly higher than DLW for the rest groups and slightly lower for flight. Nagy (71) notes that 1 to 2 half-lives of ^{18}O elimination from peak enrichment is an ideal washout to estimate metabolic rate, with errors being higher with less washout. The elimination of ^{18}O for our flight birds was close to, but significantly different, from one half-life (δ^{18}O elimination: 440 ± 110 vs. 547 ± 104 for one half-life; t = −3.14, df = 38, $P = 0.003$), but rest birds showed significantly less washout (δ^{18}O elimination: 147 ± 35.6 vs. 577 ± 61.6 for one half-life; t = −23.45, df = 28, $P < 0.001$), so there may be greater error in the DLW estimate for rest birds. Despite ^{18}O turnover less than one half-life, this method has been successful for other bird flights of approximately 6 h and is likely to be relatively accurate (48).

Using the geolocator data from 20 blackpoll warblers in the study by Deluca et al. (4), and integrating the minimum and maximum flight time and distance from the uncertainty in the data, we estimate that the birds in that study flew for 62.4 h on average (range: 42 to 102 h). Flight simulations predicted that blackpoll warblers from our study would fly for 98 ± 7 h and end their flights at 8.7 ± 0.3 g, with a minimum estimate of 6.6 g. Blackpoll warblers arriving on the coast of South America during fall migration often arrive emaciated (72), with Nisbet et al. (14) estimating that 7.9 g appeared to be the lowest limit for fat-free mass in blackpoll warblers arriving in Venezuela and Bayly et al. (27) observing the lowest mass upon arrival in Colombia at 8.3 g. In our study, we allowed three captive blackpoll warblers to fly until they voluntarily ended the flights. In the resulting flights lasting up to 28 h, each of these birds still had substantial (at least 3 g) fat remaining, but we observed slightly concave flight muscles and a final fat-free body mass of at least 9.6 g. Flight in a wind tunnel may consume more energy than wild flights with steady, consistent wingbeats (23), but by voluntarily ending flights earlier than predicted with ample fat reserves but concave flight muscle, we may find that factors other than fat mass, such as protein or water, may be important for determining flight duration. This is consistent with recent evidence from Bayly et al. (27) that out of over 1,000 blackpoll warblers captured after arriving in Colombia, only 14% and 21% of birds were fat depleted in 2017 and 2018, respectively, while 87% of the birds had depleted flight muscles (muscle scores of 1 or lower).

We found remarkably similar use of fuels in these short- and long-distance migratory warblers. Despite the difference in mass-specific metabolic power, both species used fat consistently and lost substantially more fat-free mass early in flight. When allowed to fly to voluntary completion, blackpoll warblers ended their flights with ample fat stores, but evidence of fat-free mass depletion, suggesting that protein may be a critical, dynamic, and overlooked fuel for determining the capabilities of migratory birds.

Ethics. All procedures in this study were approved by the University of Massachusetts Amherst Institutional Animal Care and Use Committee (2015-0019) and the Western University Animal Use Subcommittee (2010-216), and birds were collected under Canadian Wildlife Service permit #CA0256 issued to Dr. Christopher Guglielmo.

Data, Materials, and Software Availability. All study data are included in the article and/or *SI Appendix*.

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