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Aminopeptidase-N modulation assists lean mass anabolism during refuelling in the white-throated sparrow

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Songbirds meet the extreme metabolic demands of migration by burning both stored fat and protein. However, catabolizing these endogenous tissues for energy leads to organ atrophy, and reductions in gastrointestinal tissue can be as great as 50% of the pre-flight mass. Remarkably, during stopover refuelling birds quickly regain digestive mass and performance. Aminopeptidase-N (APN) is a brush-border enzyme responsible for late-stage protein digestion and may critically assist tissue reconstruction during the stopover, thus compensating for reduced gut size. We hypothesized that birds recovering from a fast would differentially upregulate APN activity relative to disaccharidases to rapidly process and assimilate dietary protein into lean mass. We fasted 23 wild-caught migratory white-throated sparrows (*Zonotrichia albicollis*) for 48 h to mimic mass reductions experienced during migratory flight and measured intestinal APN activity before the fast, immediately after the fast, and during recovery at 24 h and 48 h post-fast. Total fat mass, lean mass and basal metabolic rate were measured daily. We show that fasted birds maintain APN activity through the fast, despite a 30% reduction in intestine mass, but during refuelling, APN activity increases nearly twofold over pre-fasted individuals. This suggests that dynamically regulating APN may be necessary for rapid protein reconstruction during the stopover.

1. Introduction

It has long been observed that some animals can produce rapid and sometimes reversible changes to their physiology and morphology, a process now described as phenotypic plasticity, to compensate for challenges resulting from variable environments [1,2]. When there is a mismatch between environmental conditions such as food availability or daily maximal temperatures, and an organism's physiological capacity, it may be able to alter its phenotype to better perform in such an environment. Evolutionary biologists have made substantial efforts to tease out the extent by which phenotypic flexibility may influence evolution [3,4], and to this end, it is essential to identify unique ecologically relevant models where flexible trait responses of organisms can provide opportunities to define mechanistic links between individual performance limitations, phenotypic responses and real-world outcomes.

The annual migration between wintering and breeding grounds is one such challenge, where birds exhibit dramatic flexibility with respect to multiple phenotypic traits. To meet the energetic demands of flight, birds must first deposit, then burn, both fat and protein reserves to complete the journey [5–7]. This strategy may supplement gluconeogenesis or offset water loss incurred during non-stop flights over ecological barriers which prohibit the animals from stopping and eating or drinking [8–10].

Birds display drastic flexibility with respect to the digestive system before and after the migratory flight [11,12]. It is well documented that protein catabolism in flight results in dramatic lean mass reductions in muscle and organ tissue. In a real-world scenario, a bird can experience a 50% gut mass reduction during a flight and subsequently incur losses in digestive performance compared to pre-flight performance [13–16]. From shorebirds to small passerines, examples of protein degradation during flight have been well established by field observations, yet the limits of protein catabolism and the extent to which birds are able to compensate for the resulting performance deficits are still not clear [17].

Up to 20% of daily metabolic expenditure goes towards maintaining the digestive system, which has significant implications for the energy budget of actively migrating birds [18,19]. One possible reason for catabolizing the gut before and during migration may be to increase energy allocated to the mechanics of powered flight instead of maintaining digestive ability during a period of life history when the animal has no access to food [20]. Additionally, birds may catabolize metabolically active tissue, such as skeletal muscle and organ tissue, to reduce energetic costs during migratory stopover refuelling. In the context of migration, protein catabolism as a trait may be beneficial as a means of balancing the in-flight water and energy budgets, and may also be the important determinant of flight range. It is then expected that whole-animal performance, as indicated by metabolic rate, may be impacted from such drastic reduction of metabolically active lean mass. Apart from the possibility of direct lethal effects on the animal owing to the presumed loss of function associated with such dramatic loss of organ mass, the migratory pace could also be constrained by sub-lethal effects on tissue function. Ultimately, extending the time spent refuelling during migratory stopover increases both lethal and sub-lethal risks for birds as they make their journey to breeding grounds [21].

Arriving at stopover refuelling sites after a migratory flight, birds first rebuild lean mass, including skeletal muscle, kidneys, liver, heart and intestines, before they are able to replenish lipid reserves needed to continue their journey [22–25]. Central to this reconstruction is the ability to break down ingested protein into amino acids that can be absorbed in the small intestine, a process reliant on enzymatic activity during the final stages of digestion. Brush-border enzymes are expressed in the villi of small intestine enterocytes to process dietary macromolecules and damage to the epithelial structure of the small intestine, as frequently documented as a result of the long-distance flight, could reduce brush-border enzyme activity [26].

Aminopeptidase-N (APN) is a brush-border enzyme integral to the digestion of dietary protein and cleaves peptides during the final stages of protein digestion. There is evidence in birds such as domestic chickens (*Gallus gallus)*, juvenile mallards (*Anas platyrhynchos*) and Canada geese (*Branta canadensis*), that APN is dynamically increased when fed high-protein diets over periods of weeks or months [27,28]. In passerines, Caviedes-Vidal *et al*. [29] showed that enzyme modulation in house sparrows (*Passer domesticus)* also responds to diet switching under similar time periods. Brush-border disaccharidases are also regulated to match substrates in diet, (as in [29] with sucrase and maltase activity modulated in a variety of songbird species such as pine warblers (*Dendroica pinus*), flowerpiercers and hummingbirds [30,31]. These have been valuable inquiries into digestive plasticity as a trait present in birds and have opened up the possibility of linking flexible digestive phenotypes to whole-animal performance during challenging life cycle stages. However, it is still unclear how quickly and to what degree adult migratory passerines may adjust their digestive phenotype to meet the physiological demands of long-distance flight.

In the context of migratory songbirds, the ability to selectively increase APN activity relative to maltase or sucrase during periods of protein anabolism to reverse organ atrophy, rather than solely as a function of diet composition would prove to be advantageous during stopover refuelling. This study seeks to provide evidence that digestive enzymatic regulation after the long-distance flight is regulated in response to anabolic requirements for protein, rather than a diet, to assist the reconstruction of lean mass on an ecologically relevant time scale, which has not been demonstrated to date. We investigated whether digestive enzymes can be dynamically and quickly regulated in response to experimental depletion of body mass that parallels changes birds undergo during migration. We predicted that (i) birds would show significantly decreased intestinal mass and APN activity as a result of fasting in combination with overall reductions of lean mass; in addition (ii) APN activity would rapidly increase over the course of 48 h of refuelling to assist rapid anabolism of lean tissue; and (iii) reductions of basal metabolic rate (BMR) would be incurred after fasting owing to reductions of metabolically active tissue mass.

2. Methods

(a) Capture and housing

White-throated sparrows (*Zonotrichia albicollis*) were selected as the focal species for this experiment as they are a migrant that breeds in the boreal forests of Canada and winters in the southern United States and northern Mexico [32]. Migratory birds were caught using standard mist-netting techniques on the periphery of the University of Massachusetts-Amherst campus (WGS84-Projected Coordinate 42.3948, −72.5168) in September and early October 2015 (Massachusetts Division of Fisheries & Wildlife permit no. 194.15SCB; USFWS permit MB65968). Birds were kept on a light cycle synchronized weekly with the natural light cycle at the date of capture initially, until it reached 12 L : 12 D at which time it was maintained. Birds had ad libitum access to water and a diet of ground Mazuri™ Small Bird Diet mixed with standard white millet. Birds were also supplemented with meal worms, grit and egg food three times per week per standard captive animal care protocol. Prior to the start of the experiment, health checks were completed weekly to ensure captivity had no negative effects on the birds as well as to ensure that birds retained optimal body composition prior to the experiment. All procedures were approved by UMass IACUC protocol no. 2015–0019

(b) Experimental procedure

White-throated sparrows were distributed randomly into four treatment groups (control $n = 8$, fasted $n = 5$, 24 h recovery $n = 5$, and 48 h recovery $n = 5$; figure 1). No significant differences in mean mass or size (as standardized by tarsus length) existed between treatment groups at the start of the experiment. To account for effects of handling stress, the positive control group was handled as frequently as the other groups but was not subject to fasting at any point, and this group was euthanized at the conclusion of the experiment.

We performed a pilot trial to determine the rate of fat loss for a sparrow of comparable size (*P. domesticus*) which allowed

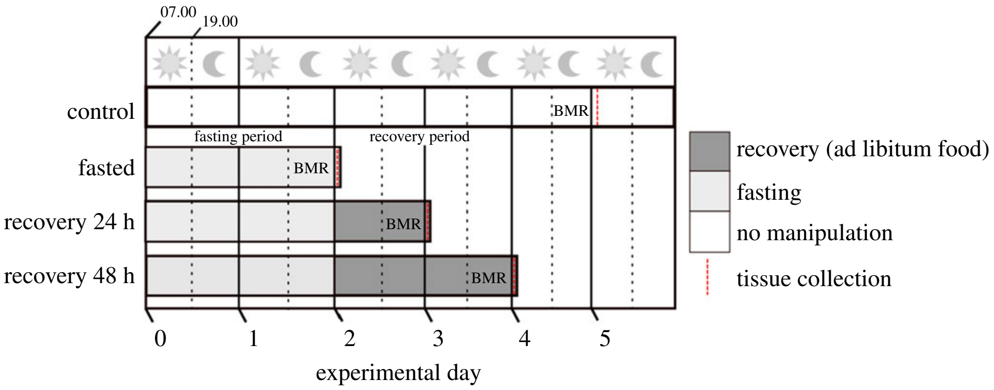

Figure 1. Four experimental groups underwent distinct fasting and recovery treatments concurrently. Light-fill indicates the fasting period for three groups (recovery 48 h $n = 5$, recovery 24 h $n = 5$ and fasted $n = 5$) which began at 07.00 on day '0'. Dark fill indicates the time period which birds had ad libitum access to food and water during a refuelling period; groups recovery 48 h and 24 h were given access to food at 07.00 on day 2. The control group ($n = 4$) had ad libitum access during the entire duration of the experiment. Dashed lines indicate the daily photo period split with lights on at 07.00 and dark at 19.00 and groups entered respirometry at dark on their scheduled days. Tissue collection began within 30 min of birds exiting respirometry the following morning (red dashed line). (Online version in colour.)

us to confidently determine the required initial body condition necessary for birds to endure a fast of 48 h without completely depleting fat stores or any risk of mortality. Birds that underwent fasting (groups: fasted, 24 h recovery and 48 h recovery) were closely monitored throughout the fast. Groups which completed the fasting period had no access to food, but had access to water, ad libitum. Birds that were in the recovery groups were given ad libitum access to seed, millet, egg food, meal worms and water after the completion of the fast. Diets before and after the fast were the same as outlined above.

(c) Body composition and metabolic rate

Fat mass, lean mass and total body water were quantified *in vivo* with the use of a non-invasive quantitative magnetic resonance (QMR) body composition analyser (Echo MRI, TX USA) which has been shown to be accurate in small birds [17] and allows for repeated measurements within individuals. Repeated measurements of body composition allow for fine-scale resolution of body composition changes during the entire experimental course as well as aiding in monitoring the health of subjects during the entirety of the experiment. All birds were scanned each morning during the experiment, before and after respirometry, and immediately prior to sampling. Post-respirometry QMR scans were used to compare the difference in total, fat and lean mass among experimental groups immediately prior to tissue collection. For our body composition analysis, experimental mass changes (total, fat and lean masses) are calculated as the individuals' difference between the initial post-absorptive mass (the morning of the start of the experiment) subtracted from post-respirometry mass at the time of dissection (recorded at one of four time points, depending on the experimental group; figure 1). This allows us to see individual-level effects of fasting and recovery, as well as allowing for comparisons of experimental group means for mass change. We also calculate the overnight mass loss in our analysis, which is the pre-BMR mass subtracted from the post-BMR scan. This allows us to compare differences in overnight fuel use among groups as a result of fasting and recovery.

$\dot{V}O_2$ and $\dot{V}CO_2$ were measured using standard flow-through respirometry techniques overnight between the hours of 20.00 and 08.00. These measurements were taken under standard BMR conditions thermoneutral environment ($T_a = 30°C$) and during rest-phase in non-moulting or breeding adults). The use of multiplexing allowed for the data collection on a total of six birds per night. Excurrent CO_2 and H_2O were measured using an LI-840A (Licor, Lincoln NB USA), and O_2 was measured using an FC-10 oxygen analyser (Sable Systems, Las Vegas NV USA). Data were collected using EXPEDATA software (Sable Systems, Las Vegas NV USA). $\dot{V}O_2$ and $\dot{V}CO_2$ and $\dot{V}H_2O$ were calculated using standard equations for push respirometry found in Lighton [33]. BMR was taken as the lowest 2 min of $\dot{V}O_2$ during the run. All birds within a given experimental group were measured on the same night.

(d) Tissue collection

At the conclusion of experimental intervals, birds were humanely euthanized by isoflurane overdose in combination with cervical dislocation. All organs were isolated and put on ice-cold saline within 5 min of time of death. Pectoralis were quantitatively excised and flash-frozen in liquid N_2 to be used in complimentary studies. Sternum and tarsus morphometrics were measured after quantitative dissection. Sections of the intestine, as well as other organs (for separate studies), were flash-frozen in liquid nitrogen for enzyme analysis (below). The small intestine between the ileum and the duodenum was sectioned, blotted dry and weighed. The length was determined using digital calipers. The excised small intestine was cut in half, and the distal-most and proximal-most 2–4 mm of intestine was removed and fixed in 10% neutral-buffered saline (Sigma Aldrich). The remaining distal and proximal sections were flash-frozen for enzyme analysis. We must note that for the first four dissected birds, the intestinal tissues were extracted in a manner deviating from the protocol above, thus morphometric and enzymatic analyses would not be comparable across all birds. These birds belonged to a control group which was designated as a 'pre-experimental' group, that is, birds which were to be euthanized prior to the start of any experimental manipulation on the other cohorts. We believe it is best to exclude these animals so as to avoid erroneous analysis. This error, however, is independent to the measurements of metabolic rate and whole-animal mass change, as those were quantified on the organism *in situ* prior to dissection.

(e) Tissue homogenization

Intestinal tissue was homogenized using a NextAdvance® Bullet™ Storm 24 blender. After initial excision, the small intestine was divided into proximal and distal portions which were then prepared for homogenization by being further partitioned into 50 mg sections and added to nine volumes of 350 mM Hepes/1 mM Mannitol buffer in a 1.5 ml microcentrifuge tube. The bead mill blender was housed in an incubator set to

4°C to prevent enzymatic degradation occurring during homogenization. The homogenate was partitioned into three separate aliquots and frozen at −80°C until analysis.

(f) Enzyme assays

Using L-alanine-p-nitroanalide as a substrate, APN was assayed as described in Caviedes-Vidal *et al.* [29] and Chediack *et al.* [34]. Briefly, 10 µl of the homogenate was pipetted in duplicate into a 96-well plate. Using a Gen5™ plate reader (BioTek, Vt, USA), a 290 µl of assay buffer (2.0 mM L-alanine-p-nitroanalide substrate in 0.2 M phosphate buffer (NaH_2PO_4/Na_2HPO_4, pH 7 at room temperature)) was added to each well and was incubated for 10 min at 39°C while kinetically measuring absorbance at 384 nm. We empirically determined the extinction coefficient of nitroanlide to be 12.31 mmol^{-1} cm^{-1} in the conditions of this assay. Enzyme activity was calculated as the maximum 45 s of the reaction in μmol $*$ min^{-1}. The disaccharidases, maltase and sucrase were assayed similarly, as described by Martinez del Rio [35] and Caviedes-Vidal *et al.* [29]. Enzyme activity is standardized per gram of wet mass.

(g) Data analysis

Changes in body composition from the initial state, overnight mass loss, organ mass, BMR and enzyme activities among treatment groups were compared using ANOVA ($\alpha = 0.05$) in statistical software R and graphic plots were generated using the package ggplot2. Tukey's HSD test was performed for scenarios where intra-group comparisons were relevant.

3. Results

(a) Experimental mass changes

At the start of the experiment, average mass across all individuals was 30.85 g and did not significantly vary among groups ($F_{3,15} = 0.2821$, $p = 0.8375$). Neither fat mass (mean 7.56 g) nor lean mass (mean 19.19 g) varied significantly among groups at the start of the experiment (fat: $F_{3,15} = 0.672$, $p = 0.582$; lean: $F_{3,15} = 0.2522$, $p = 0.8585$).

There were significant mass changes among groups ($F_{3,15} = 9.7305$, $p < 0.001$; figure 2). Relative to initial mass, the fasted group had the largest reductions in total mass at the time of tissue collection, losing an average of 5.9 g mass, or roughly 21% of body mass which was significantly greater than mass loss when compared to the control group which experienced a 7% reduction in total mass (Tukey HSD: fasted–control, $p = 0.0009$). Additionally, the other two groups which underwent 48 h of fasting (recovery 24 h and recovery 48 h) had significantly greater reductions in total mass compared to the control group (Tukey HSD: recovery 24 h–control, $p = 0.0030$; recovery 48 h–control $p = 0.0036$) and retained a mass deficit through the conclusion of the experiment. These groups experienced a 19% and 18% reduction in mass, respectively.

We found significant differences in lean mass changes among groups at the time of sampling ($F_{3,15} = 9.079$, $p = 0.0011$; figure 2). The fasted group lost an average of 3.05 g (16%) of lean mass from starting values, which was significantly greater than all other experimental groups (Tukey HSD: control–fasted, $p = 0.006$; recovery 24 h–fasted $p = 0.005$; recovery 48 h–fasted, $p = 0.001$). There were no differences in lean mass change between the recovery 24 h, recovery 48 h and control groups ($p > 0.90$ for all Tukey pairwise comparisons) as these groups lost an average of 3% lean mass.

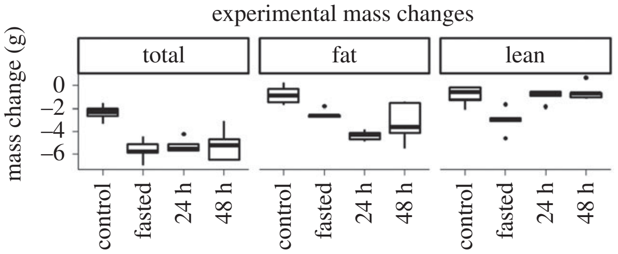

Figure 2. Experimental mass changes. Individuals were measured for total, fat and lean mass prior to and after an induced fast. The three groups which underwent the fast all had significantly greater reductions in total mass by the end of their respective experimental periods. However, only the fasted group (which was sampled without the opportunity to refuel after the fast) had significantly greater lean mass reductions compared to the control group. Recovery 24 h and 48 h groups continued catabolizing fat through recovery, but did not have significantly less lean mass reduction compared to the control, indicating that protein was replenished in as little as 24 h after the fast.

We recorded significant differences in the changes in fat mass among groups ($F_{3,15} = 9.0441$, $p = 0.0011$; figure 2) in response to fasting. However, there was not a significant difference in the reduction of fat mass between the control and fasted group (Tukey HSD: control–fasted, $p = 0.12$). After 24 h of recovery, birds had an average of 2.26 g less fat mass than did the fasted group (Tukey HSD: fasted–recovery 24 h, $p = 0.053$), indicating that fat catabolism outpaced anabolism during the course of recovery. Both the recovery 24 h and 48 h groups lost significantly more fat mass than did the control group (Tukey HSD: recovery 24 h–control, $p = 0.0006$, recovery 48 h–control, $p = 0.016$).

(b) Body composition changes during metabolic testing

Though fasting did not have an effect on total mass loss overnight during metabolic testing ($p > 0.05$), the relative proportions of fat and protein changed among treatment groups. There were significant differences in the amount of lean mass loss that occurred during metabolic testing ($F_{3,19} = 3.672$; $p = 0.036$). The fasted group lost significantly more lean mass than the control group ($p < 0.05$), losing on average 1.7 g during the overnight test, which was more than twofold greater than lean mass reductions in control groups. Furthermore, the fasted group lost less fat mass overnight than did the recovery 24 h group (ANOVA: $F_{3,17} = 3.1909$; $p = 0.047$; Tukey HSD: fasted–recovery 24 h, $p = 0.0409$).

(c) Intestinal mass changes

There were significant differences in gut mass among groups ($F_{3,15} = 5.239$, $p = 0.0113$) and differences in length ($F_{3,15} = 3.029$, $p = 0.06$). The fasted group had a 24% reduction in intestine mass in comparison to the control ($p < 0.05$) and the recovery 48 h group had significantly recovered intestine mass relative to the fasted group (fasted–recovery 48 h; $p = 0.009$), and actually increased intestine mass by 10% over the control group, although this was not significant (Tukey HSD: control–recovery 48 h $p = 0.67$; figure 3). Pairwise comparisons between the recovery 24 h group and the rest of the treatments yielded no significant differences in mass.

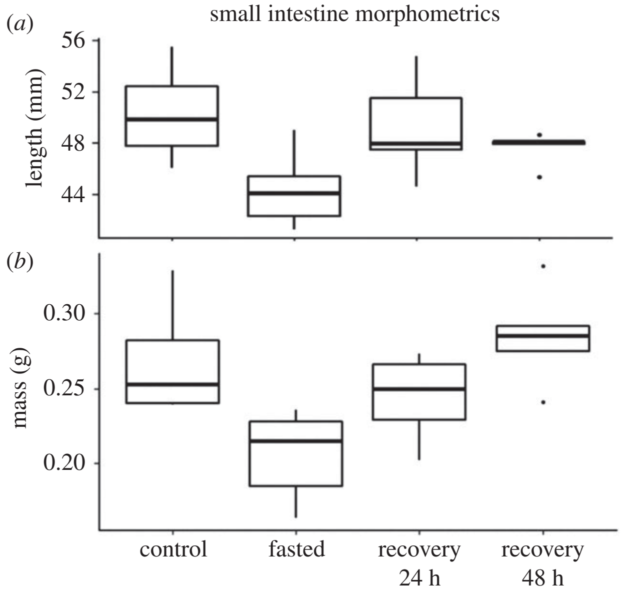

Figure 3. Intestinal mass and length changes. The fasted group had significantly smaller length and mass compared to the control, a result of 48 h of fasting. After 24 h of recovery, both the recovery 24 and recovery 48 h groups regained intestinal length matching that of the control (*a*). After 48 h of recovery, the recovery 48 h group had on average 10% more small intestine mass compared to the control group (*b*).

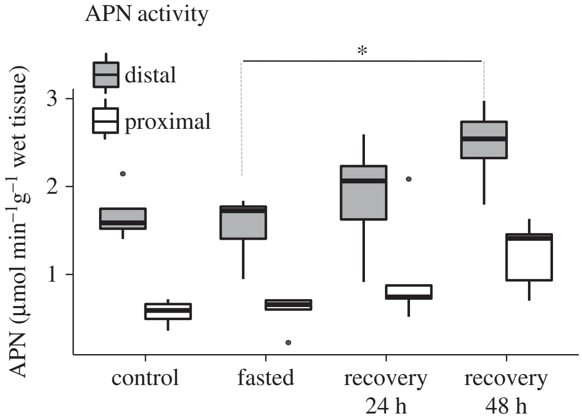

Figure 4. Activity rates of APN in the distal and proximal sections of the small intestine at time of dissection. There were no differences in activity between the 'control' and 'fasted' groups at the time of sampling. However, after the fasted birds were allowed to refuel, individuals in the 'recovery 24' and 'recovery 48' groups increased their APN activity. The 'recovery 48 h' group had significantly higher APN activity rates than the 'fasted' group and near-significant increase over the 'control' group.

(d) Enzyme activity

There were significant differences in APN activity in distal sections and near-significant differences in proximal sections of intestine among groups (distal- $F_{3,15} = 3.752$, $p = 0.03$; proximal; $F_{3,15} = 3.091$, $p = 0.056$; figure 4). We observed no reduction in APN enzyme activity in response to the 48 h fast (Tukey HSD: control–fasted $p = 0.962$). Yet, after refuelling, birds in the recovery 48 h group had significantly higher APN activity compared to the fasted group (Tukey HSD recovery 48 h–fasted; $p = 0.03$) and near-significant increase over the control group ($p = 0.09$). We found no significant differences in maltase activity among groups (ANOVA; $F_{3,15} = 4.008$, $p = 0.754$). We were not able to detect the sucrase activity in either proximal or distal small intestine tissue.

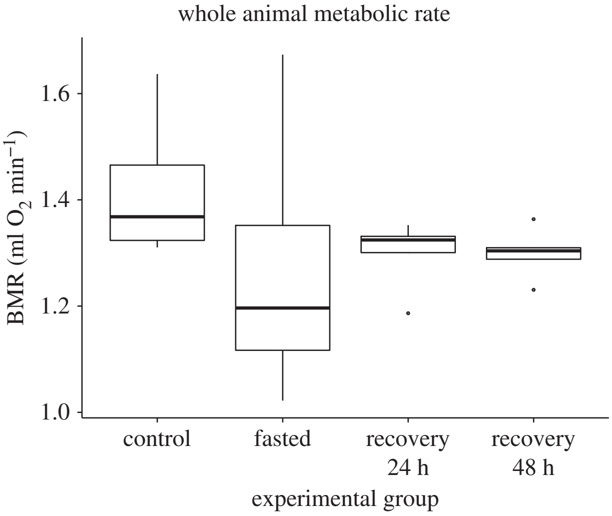

Figure 5. Whole- animal basal metabolic rates of experimental groups. Fasting had no effect on metabolic rate despite significant lean mass reductions resulting from experimental manipulation.

(e) Metabolic rates

Despite large fluctuations in lean, fat and total mass over the course of fasting and refuelling, we did not document any significant differences in whole-animal overnight resting metabolism among experimental groups ($F_{3,19} = 0.7943$, $p = 0.512$; figure 5). Whole animal $\dot{V}O_2$ was 10% higher than allometrically predicted BMR [36], indicating our resting conditions approximated standard conditions commonly used to measure BMR. We directly calculated respiratory quotient (RQ) which did not significantly vary between groups and had a mean value of 0.694 (±.004; $n = 23$).

4. Discussion

We present evidence that white-throated sparrows defend APN activity despite significant reductions in intestine mass and size during a fast intended to mimic migratory flight. After recovery from the fast, birds improved their capacity to process protein in the gut via a twofold increase in intestinal APN activity during recovery, relative to controls, which then enables rapid lean mass anabolism. Our experimental design is, to our knowledge, the first of its kind to combine the measurement of brush-border enzyme activity with quantitative body composition data to better understand how the plasticity of digestive physiology may assist refuelling in a migratory passerine. While previous experiments have shown that birds possess great digestive flexibility when exposed to diet switching, notably [28,29,37,38], we have evidence of enzyme modulation to meet the needs of refuelling and mass gain in an ecologically relevant time scale. Independently of diet quality, white-throated sparrows increase APN activity to optimize protein digestion efficiency in as little as 24 h after a fast.

Rott *et al.* [39] demonstrate digestive enzyme modulation occurs in captive nestlings in response to novel diets through the course of nestling development, to demonstrate developmental plasticity in response to diet composition through this important life stage. By contrast, our study demonstrates rapid flexibility in gut enzyme activity in adult birds, where APN is upregulated rapidly during a period of high-protein anabolism, thus facilitating protein assimilation despite dramatically reduced gut size overall. Our experiment demonstrates the capacity for dramatic changes in the digestive phenotype that is specifically decoupled from both organismal development and diet switching.

Our findings demonstrate that songbirds have the capacity to modulate their digestive enzymes during migratory stopover when the need to rebuild specific tissues is high, analogous to Rott *et al*. [39]. Yet, by contrast, our results indicate that adult migratory passerines retain the capacity for rapid plasticity of brush-border enzymes that is not contingent upon diet. Migratory bird physiologists have long sought explanations for birds' rapid deposition of fat and muscle after long-distance flight and here we provide a physiological mechanism which may assist rapid refuelling. In a real-world refuelling scenario, wild-living birds may further capitalize on this trait by modifying behaviour resulting in higher amino acid intake (i.e. by selecting protein-rich food sources) to exploit variable resources during stopover refuelling. While prior studies have clearly demonstrated the importance of digestive plasticity in response to diet alone, the results of the present experiment provide a novel perspective: migratory birds can increase capacity for protein digestion even in the absence of dietary changes. We propose that such a flexible digestive phenotype in combination with birds' likely preference for high-protein food sources at stopover would accelerate protein assimilation during migratory refuelling. Notably, the data also show a clear biphasic mass gain pattern during recovery. Initially, birds augment APN activity to prioritize quick assimilation of dietary protein which is necessary to rebuild the gut and lean body mass, and subsequently begin to deposit fat mass. Our whole-animal body composition data reveals that birds fully recovered lean mass to pre-fasting levels within 48 h, but did not fully replenish fat reserves by the end of our experiment, providing further evidence that birds prioritize the reconstruction of digestive machinery prior to allocating fuel for the flight. In fact, the continued decline in fat mass that occurred during the first 24 h of recovery indicates that exogenous energy assimilation was insufficient to meet the requirements of recovery for these birds, which may be owing to a digestive constraint imposed by reduced intestine mass.

We propose that this flexible trait may be adaptive in the context of migratory fuelling, where birds must remodel the morphology and physiology of the intestine to accommodate new diets at each stopover along the migratory journey. Rather than arriving to stopover grounds after a long duration flight with digestive physiology optimized to pre-migratory feeding grounds, catabolizing the intestine in flight may allow a more rapid remodelling of the gut to suit the new habitat and diet encountered after a flight during refuelling, preventing a mismatch between pre-flight physiology and food availability at the stopover. In combination, this physiological strategy to rapidly change digestive capacity results in the ability to process bulk dietary protein without the cost of maintaining a large and metabolically expensive gut throughout the entirety of migratory flight [13], because a highly optimized gut is necessary to replenish lean mass quickly during stopover refuelling, and may be essential to reduce time spent during migratory stopover.

Birds that underwent fasting lost more lean and fat mass than did control groups. There are concerns about the differences between tissue catabolism in-flight and those incurred during a low-energy fast, chiefly regarding possible differences in fuel source mixture under high- versus low-energy states [40]. However, our experimental design induced mass reductions in captive birds which have been commonly observed in free-living animals after migration [10,14,41]. Despite these large reductions in lean mass and organ size, we did not document significant differences in overnight resting metabolic rates, contrary to our predictions and other observations [42–44]. Ellis & Gabrielson [45] argue that the long-held definition of BMR may not always be comparable across populations or even individuals and reported values are not always standardized by rest or active-phase. We document overnight resting RMR, collected under standard conditions used to measure BMR, which were on average 10% higher than predicted values for birds of similar size. Because birds may be in migratory disposition, we are hesitant to classify our measurements of metabolism as true BMR. Also notable in the context of Barcelo *et al*. [46], birds increased basal and summit metabolic rates to endure cold challenges, but such performance was not linked to total skeletal muscle mass, rather digestive organ size.

BMR, while easily obtained with high precision, is still a trait influenced by circannual rhythms, especially in birds as seasonally plastic as migratory birds [47]. While measuring metabolism under BMR conditions may be a useful measurement in many contexts, we argue that the lowest measurements of rest-phase metabolism over 8–12 h may not be meaningful in the framework of birds in active migratory disposition, particularly because birds in migratory disposition may violate the assumptions of BMR conditions. The transition to a breeding phenotype is regulated by a cascade of hormonal interactions resulting in metabolically intensive periods such as gonad formation and moult regiments [48,49] which may negate BMR as the most illuminating metric of metabolism in the context of migratory refuelling. As a result, it may be that migratory birds have lower total overnight energy expenditure after flights rather than simply lowering their metabolic floor for a few minutes overnight, a phenomenon not captured solely thought the measurement of the lowest instance of metabolic rate overnight. With the use of rapid multiplexing which is now becoming more available, high-resolution metabolic phenotyping will be useful elucidate this problem.

Early attempts to link body composition changes in response to endurance flight have shown that fat is the dominant component of the fuel mixture in migratory birds [5]. By providing gluconeogenic precursors and Krebs cycle intermediates during fatty acid metabolic oxidation, lean mass catabolism assists critical metabolic pathways under high energetic demand and, as a result, it has been conventionally thought that birds switch over to protein as a secondary fuel source once the fat has been sufficiently used [50–53] Yet, the evidence is emerging that birds can catabolize protein simultaneously to fat metabolism under certain environmental conditions such as water stress [6,8,10]. More recently, protein catabolism has been documented in controlled laboratory environments in which wild-caught passerines flown in wind tunnels for long durations catabolize protein prior to depleting all fat reserves [10]. Here, we present experimental evidence that birds preferentially catabolize significant amounts of protein mass *prior* to exhausting endogenous lipid reserves during fasting as well. Much like free-living birds, these birds did not completely catabolize all fat prior to breaking down lean tissue [14]. After 48 h of fasting, birds lost an average of 3.03 g or 15% of protein mass relative to their starting body composition yet, still had 4.87 g of fat remaining, representing 19.75% of their total body mass.

While our results add greatly to the understanding of migratory stopover physiology, we believe there is still much foundational data to be collected on stopover digestive physiology as it relates to body composition, organ mass and functionality. For instance, given our finding that the modulation of brush-border enzymes occurs in response to physiological state, investigation of the underlying regulatory genetic or hormonal mechanisms responsible may shed light on the link between well documented developmental plasticity processes and our findings in adult organisms. Additionally, flying vertebrates have smaller guts than similarly sized non-flying vertebrates to reduce ballast during flight, and there is much research into how their use of paracellular absorption can compensate for digestive limitation owing to reduced gut size [26]. While there is evidence of changes to paracellular absorptive performance in short time scales, notably [54], no studies quantify this dynamic phenotype in the context of mass anabolism. Furthermore, studies suggest that paracellular absorption is beneficial for immediate glucose synthesis, yet little is known if it also may be beneficial for rapid amino acid shuttling, particularly after challenging life-history events such as migration in songbirds when there is substantial need to rebuild tissues. Without maltase, birds can still rapidly incorporate saccharides via paracellular absorption, which may explain why we don't see the same patterns of rapid increases in disaccharidases [55]. However, APN is necessary to cleave large peptide chains before further transportation in the lumen [56]. Owing to the seasonality of migratory physiology, especially one that is well-optimized to incorporate exogenous amino acids into tissues, we also propose future research into measuring the flexibility of this trait throughout the annual life cycle. Diet switching studies can be particularly helpful in defining this trait, as pre/post migratory, breeding and wintering phenotypes can be induced in captive birds.

Ethics. Migratory birds were caught using standard mist-netting techniques on the periphery of the University of Massachusetts-Amherst campus (WGS84-Projected Coordinate 42.3948, -72.5168) in September and early October 2015 (Massachusetts Division of Fisheries & Wildlife permit no. 194.15SCB). All procedures were approved by UMass IACUC protocol no. 2015-0019.

Data accessibility. Data available as part of the electronic supplementary material. Additionally, we have included an R markdown that details our statistical approach. In the event requesting parties cannot obtain access to available data online, please request data directly from the corresponding author.

Authors' contributions. M.G. and A.R.G. contributed to conceptualization, hypotheses development and experimental design. M.G. collected the data with assistance from M.G.R. and J.D. M.G. performed formal analysis and drafted the manuscript with input from A.R.G., M.G.R. and J.D.

Competing interests. We declare we have no competing interests.

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References

1. West-Eberhard MJ. 1989 Phenotypic plasticity and the origins of diversity. *Annu. Rev. Ecol. Syst.* **20**, 249–278. (doi:10.1146/annurev.es.20.110189.001341)
2. Scheiner SM. 1993 Genetics and evolution of phenotypic plasticity. *Annu. Rev. Ecol. Syst.* **24**, 35–68. (doi:10.1146/annurev.es.24.110193.000343)
3. Woods HA, Harrison JF. 2002 Interpreting rejections of the beneficial acclimation hypothesis: when is physiological plasticity adaptive? *Evolution* **56**, 1863–1866. (doi:10.1111/j.0014-3820.2002.tb00201.x)
4. Hendry AP. 2015 Key questions on the role of phenotypic plasticity in eco-evolutionary dynamics. *J. Heredity* **107**, 25–41. (doi:10.1093/jhered/esv060)
5. Jenni L, Jenni-Eiermann S. 1998 Fuel supply and metabolic constraints in migrating birds. *J. Avian Biol.* **29**, 521–528. (doi:10.2307/3677171)
6. Gerson AR, Guglielmo CG. 2013 Energetics and metabolite profiles during early flight in American robins (*Turdus migratorius*). *J. Comp. Physiol. B* **183**, 983–991. (doi:10.1007/s00360-013-0767-y)
7. Klaassen M, Hoye BJ, Nolet BA, Buttemer WA. 2012 Ecophysiology of avian migration in the face of current global hazards. *Phil. trans. R. Soc. Lond. B* **367**, 1719–1732. (doi:10.1098/rstb.2012.0008)
8. Gerson AR, Guglielmo CG. 2011 Flight at low ambient humidity increases protein catabolism in migratory birds. *Science* (*New York, N.Y.*) **333**, 1434–1436. (doi:10.1126/science.1210449)
9. Jenni-Eiermann S, Jenni L. 2001 Postexercise ketosis in night-migrating passerine birds. *Physiol. Biochem. Zool.* **74**, 90–101. (doi:10.1086/319306)
10. Groom DJ, Deakin JE, Lauzau MC, Gerson AR. 2019 The role of humidity and metabolic status on lean mass catabolism in migratory Swainson's thrushes (*Catharus ustulatus*). *Proc. R. Soc. B* **286**, 20190859. (doi:10.1098/rspb.2019.0859)
11. McWilliams SR, Karasov WH. 2001 Phenotypic flexibility in digestive system structure and function in migratory birds and its ecological significance. *Comp. Biochem. Physiol. A: Mol. Integr. Physiol.* **128**, 577–591. (doi:10.1016/S1095-6433(00)00336-6)
12. McWilliams SR, Karasov WH. 2014 Spare capacity and phenotypic flexibility in the digestive system of a migratory bird: defining the limits of animal design. *Proc. R. Soc. B* **281**, 20140308. (doi:10.1098/rspb.2014.0308)
13. Bauchinger U, Biebach H. 2001 Differential catabolism of muscle protein in garden warblers (*Sylvia borin*): flight and leg muscle act as a protein source during long-distance migration. *J. Comp. Physiol. B* **171**, 293–301. (doi:10.1007/s003600100176)
14. Battley PF, Piersma T, Dietz MW, Tang S, Dekinga A, Hulsman K. 2000 Empirical evidence for differential organ reductions during trans-oceanic bird flight. *Proc. R. Soc. B* **267**, 191–195. (doi:10.1098/rspb.2000.0986)
15. Bauchinger U, Kolb H, Afik D, Pinshow B, Biebach H. 2009 Blackcap warblers maintain digestive efficiency by increasing digesta retention time on the first day of migratory stopover. *Physiol. Biochem. Zool.* **82**, 541–548. (doi:10.1086/603638)
16. Karasov WH, Pinshow B. 1998 Changes in lean mass and in organs of nutrient assimilation in a long-

distance passerine migrant at a springtime stopover site. *Physiol. Zool.* **71**, 435–438. (doi:10.1086/515428)

17. Guglielmo CG, Piersma T, Williams TD. 2001 A sport-physiological perspective on bird migration: evidence for flight-induced muscle damage. *J. Exp. Biol.* **204**(Pt 15), 2683–2690.
18. Cant JP, McBride BW, Croom WJ. 1996 The regulation of intestinal metabolism and its impact on whole animal energetics. *J. Anim. Sci.* **74**, 2541–2553. (doi:10.2527/1996.74102541x)
19. Karasov WH, Martínez del Rio C, Caviedes-Vidal E. 2011 Ecological physiology of diet and digestive systems. *Annu. Rev. Physiol.* **73**, 69–93. (doi:10.1146/annurev-physiol-012110-142152)
20. Piersma T, Gill Jr RE. 1998 Guts don't fly: small digestive organs in obese bar-tailed godwits. *The Auk* **115**, 196–203. (doi:10.2307/4089124)
21. Seewagen CL, Guglielmo CG, Morbey YE. 2013 Stopover refueling rate underlies protandry and seasonal variation in migration timing of songbirds. *Behav. Ecol.* **24**, 634–642. (doi:10.1093/beheco/ars225)
22. Carpenter FL, Hixon MA, Beuchat CA, Russell RW, Paton DC. 1993 Biphasic mass gain in migrant hummingbirds: body composition changes, torpor, and ecological significance. *Ecology* **74**, 1173–1182. (doi:10.2307/1940487)
23. Gannes LZ. 2001 Comparative fuel use of migrating passerines: effects of fat stores, migration distance, and diet. *The Auk* **118**, 665–677. (doi:10.1093/auk/118.3.665)
24. Karasov WH, Pinshow B. 2000 Test for physiological limitation to nutrient assimilation in a long-distance passerine migrant at a springtime stopover site. *Physiol. Biochem. Zool.* **73**, 335–343. (doi:10.1086/316746)
25. Munoz-Garcia A, Aamidor SE, McCue MD, McWilliams SR, Pinshow B. 2012 Allocation of endogenous and dietary protein in the reconstitution of the gastrointestinal tract in migratory blackcaps at stopover sites. *J. Exp. Biol.* **215**(Pt 7), 1069–1075. (doi:10.1242/jeb.062547)
26. Karasov WH, Pinshow B, Starck JM, Afik D. 2004 Anatomical and histological changes in the alimentary tract of migrating blackcaps (*Sylvia atricapilla*): a comparison among fed, fasted, food-restricted, and refed birds. *Physiol. Biochem. Zool.* **77**, 149–160. (doi:10.1086/381465)
27. Iji PA, Saki AA, Tivey DR. 2001 Intestinal structure and function of broiler chickens on diets supplemented with a mannan oligosaccharide. *J. Sci. Food Agric.* **81**, 1186–1192. (doi:10.1002/jsfa.925)
28. Kohl KD, Ciminari ME, Chediack JG, Leafloor JO, Karasov WH, McWilliams SR, Caviedes-Vidal E. 2016 Modulation of digestive enzyme activities in the avian digestive tract in relation to diet composition and quality. *J. Comp. Physiol. B* **187**, 339–351. (doi:10.1007/s00360-016-1037-6)
29. Caviedes-Vidal E, Afik D, Martinez del Rio C, Karasov WH. 2000 Dietary modulation of intestinal enzymes of the house sparrow (*Passer domesticus*): testing an adaptive hypothesis. *Comp. Biochem. Physiol. A: Mol. Integr. Physiol.* **125**, 11–24. (doi:10.1016/S1095-6433(99)00163-4)
30. Levey DJ, Place AR, Rey PJ, Martínez del Rio C. 1999 An experimental test of dietary enzyme modulation in pine warblers *Dendroica pinus*. *Physiol. Biochem. Zool.* **72**, 576–587. (doi:10.1086/316689)
31. Schondube JE, Martinez Del Rio C. 2004 Sugar and protein digestion in flowerpiercers and hummingbirds: a comparative test of adaptive convergence. *J. Comp. Physiol. B* **174**, 263–273. (doi:10.1007/s00360-003-0411-3)
32. Falls JB, Kopachena JG. 2010 White-throated sparrow (*Zonotrichia albicollis*). In *The birds of North America online* (ed. A Poole). Ithaca, NY: Cornell Laboratory of Ornithology. See: http://bna.birds.cornell.edu/bna/species/128 (accessed 20 October 2020).
33. Lighton JR. 2018 *Measuring metabolic rates: a manual for scientists*. Oxford, UK: Oxford University Press.
34. Chediack JG, Funes SC, Cid FD, Filippa V, Caviedes-Vidal E. 2012 Effect of fasting on the structure and function of the gastrointestinal tract of house sparrows (*Passer domesticus*). *Comp. Biochem. Physiol. A: Mol. Integr. Physiol.* **163**, 103–110. (doi:10.1016/j.cbpa.2012.05.189)
35. Martínez del Rio C. 1990 Dietary, phylogenetic, and ecological correlates of intestinal sucrase and maltase activity in birds. *Physiol. Zool*. **63**, 987–1011.
36. Withers PC. 1992 *Comparative animal physiology*, p. 95. Philadelphia, PA: Saunders College Publications.
37. Sabat P, Novoa F, Bozinovic F, Martínez del Rio C. 1998 Dietary flexibility and intestinal plasticity in birds: a field and laboratory study. *Physiol. Biochem. Zool.* **71**, 226–236.
38. Biviano AB, Martinez Del Río C, Phillips DL. 1993 Ontogenesis of intestine morphology and intestinal disaccharidases in chickens (Gallus gallus) fed contrasting purified diets. *J. Comp. Physiol. B* **163**, 508–518.
39. Rott KH, Caviedes-Vidal E, Karasov WH. 2017 Intestinal digestive enzyme modulation in house sparrow nestlings occurs within 24 h of a change in diet composition. *J. Exp. Biol.* **220**, 2733–2742. (doi:10.1242/jeb.157511)
40. Battley PF, Dietz MW, Piersma T, Dekinga A, Tang S, Hulsman K. 2001 Is long-distance bird flight equivalent to a high-energy fast? Body composition changes in freely migrating and captive fasting great knots. *Physiol. Biochem. Zool.* **74**, 435–449. (doi:10.1086/320432)
41. Bauchinger U, McWilliams SR. 2012 Tissue-specific mass changes during fasting: The protein turnover hypothesis. In *Comparative physiology of fasting, starvation, and food limitation*, pp. 193–206. Berlin, Germany: Springer.
42. Wiersma P, Nowak B, Williams JB. 2012 Small organ size contributes to the slow pace of life in tropical birds. *J. Exp. Biol*. **215**, 1662–1669.
43. Piersma T, Bruinzeel L, Drent R, Kersten M, Van der Meer J, Wiersma P. 1996 Variability in basal metabolic rate of a long-distance migrant shorebird (red knot, *Calidris canutus*) reflects shifts in organ sizes. *Physiol. Zool*. **69**, 191–217.
44. Petit M, Lewden A, Vézina F. 2014 How does flexibility in body composition relate to seasonal changes in metabolic performance in a small passerine wintering at northern latitude? *Physiol. Biochem. Zool.* **87**, 539–549. (doi:10.1086/676669)
45. Ellis HI, Gabrielsen GW. 2019 Reassessing the definition of basal metabolic rate: circadian considerations in avian studies. *Comp. Biochem. Physiol. A: Mol. Integr. Physiol.* **237**, 110541. (doi:10.1016/j.cbpa.2019.110541)
46. Barceló G, Love OP, Vézina F. 2017 Uncoupling basal and summit metabolic rates in white-throated sparrows: digestive demand drives maintenance costs, but changes in muscle mass are not needed to improve thermogenic capacity. *Physiol. Biochem. Zool*. **90**, 153–165.
47. McKechnie AE, Wolf BO. 2004 The allometry of avian basal metabolic rate: good predictions need good data. *Physiol. Biochem. Zool*. **77**, 502–521.
48. Pathak VK, Chāndolā A. 1982 Involvement of thyroid gland in the development of migratory disposition in the redheaded bunting, *Emberiza bruniceps*. *Horm. Behav.* **16**, 46–58. (doi:10.1016/0018-506X(82)90005-8)
49. Holberton RL, Dufty AM. 2005 Hormones and variation in life history strategies of migratory and nonmigratory birds. In *Birds of two worlds: the ecology and evolution of migration* (eds P Marra, E Greenburg), pp. 290–302. Baltimore, MA: John Hopkins Press.
50. Jenni-Eiermann S, Jenni L, Kvist A, Lindström Å, Piersma T, Visser GH. 2002 Fuel use and metabolic response to endurance exercise: a wind tunnel study of a long-distance migrant shorebird. *J. Exp. Biol.* **205**, 2453–2460.
51. McWilliams SR, Guglielmo C, Pierce B, Klaassen M. 2004 Flying, fasting, and feeding in birds during migration: a nutritional and physiological ecology perspective. *J. Avian Biol.* **35**, 377–393. (doi:10.1111/j.0908-8857.2004.03378.x)
52. Klaassen M, Kvist A, Lindström Å. 2000 Flight costs and fuel composition of a bird migrating in a wind tunnel. *The Condor* **102**, 444–451.
53. Jenni L, Jenni-Eiermann S, Spina F, Schwabl H. 2000 Regulation of protein breakdown and adrenocortical response to stress in birds during migratory flight. *Am. J. Physiol. Reg. Integr. Comp. Physiol.* **278**, R1182–9. (doi:10.1152/ajpregu.2000.278.5.R1182)
54. Tracy CR, McWhorter TJ, Wojciechowski MS, Pinshow B, Karasov WH. 2010 Carbohydrate absorption by blackcap warblers (*Sylvia atricapilla*) changes during migratory refuelling stopovers. *J. Exp. Biol.* **213**, 380–385. (doi:10.1242/jeb.040071)
55. Caviedes-Vidal E, McWhorter TJ, Lavin SR, Chediack JG, Tracy CR, Karasov WH. 2007 The digestive adaptation of flying vertebrates: high intestinal paracellular absorption compensates for smaller guts. *Proc. Natl Acad. Sci. USA* **104**, 19 132–19 137. (doi:10.1073/pnas.0703159104)
56. Price ER, Brun A, Caviedes-Vidal E, Karasov WH. 2015 Digestive adaptations of aerial lifestyles. *Physiology* **30**, 69–78.