ORGANISMAL BIOLOGY

Myo-inositol is a key regulator of avian metabolism: From mechanisms to seasonal behavior

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Being naturally hyperglycemic and insulin insensitive, birds maintain plasma glucose levels twice as high as mammals of similar size. Recent evidence suggests that perturbation of myo-inositol (MI) plays a role in mammalian hyperglycemic regulation. Using an integrative approach, we identify a fundamental role of MI in avian metabolism. We show that MI transporters are highly conserved across birds and that dietary MI reduces fat accumulation in Anna's hummingbirds. MI consumption by hummingbirds varies with seasonal changes in body mass, consistent with a regulatory role. Furthermore, MI enhances fatty acid oxidation in avian cells, via effects on pyruvate-dehydrogenase complexes, indicating a role in mitochondrial fuel selection. Our findings underscore the importance of MI in avian metabolism, offering insights into their evolutionary adaptations in the context of insulin insensitivity.

INTRODUCTION

Birds maintain twice the plasma-glucose concentration of mammals at equivalent body mass without developing symptoms of diabetes. Furthermore, they do so with only ~10% of mammalian insulin levels and are relatively insensitive to insulin (*1*). As a consequence, most avian species have mechanisms to enhance fatty acid (FA) transport and oxidative capacity (*2*) using pathways comparable to those in diabetic humans who are unable to process glucose efficiently and who rely more on FA oxidation.

In birds, fats are the primary fuel for endurance activity such as migratory flights, as well as for nocturnal fasting (*3*). Birds are known for their tight regulation of body mass and fat deposition (*4*, *5*), and they minimize excess body mass. Consequently, birds do not typically store substantial glycogen, which has a high mass density given its hygroscopic characteristics (*6*). Similarly, hovering and nectar-feeding birds, such as hummingbirds, are recognized for their stringent regulation of body mass, which is intricately linked to their daily nectar intake (*7*).

Recent evidence suggests that perturbations to myo-inositol (MI) play a role in hyperglycemic regulation and fat metabolism for both birds and mammals (*8*). MI is a sugar alcohol synthesized from glucose-6-phosphate and is occasionally found in nectar and fruits but is mostly regulated endogenously (*9*, *10*). While MI is not typically used for energy, it serves as a crucial mediator in signal transduction, regulating multiple metabolic processes (*11*). Specifically, MI and derivatives can act as secondary messengers downstream of insulin receptors (*9*). Via dietary supplementation, MI can reduce diabetic symptoms and hyperglycemic indices of diabetic humans and model animals such as rats and mice (*12*–*15*). Hence, a better understanding of the role of MI in metabolism could provide key insights into its abnormalities in hyperglycemic regulation and their consequentially abnormal fat metabolism.

In vertebrates, MI also participates in essential biochemical pathways of relevance to birds, including lipid metabolism (*15*, *16*) and reduction of tissue oxidative stress (*17*). It was previously found that plasma MI was significantly increased in freshly landed migrating birds during stopover (as compared to refueling individuals) and that circulating MI was positively correlated with higher body mass and higher plasma glucose (*18*). Osipova *et al.* (*19*) suggested that, in hummingbirds, there likely has been positive selection on a MI transporter (*SLC2A13*); in a frugivorous bat, there is also up-regulation (as compared to an insectivorous bat) and organ-specific differential expression of two MI transporters (*SLC5A10* and *SLC2A13* for kidneys and pancreas, respectively) (*20*).

Given a potential connection between MI levels and fat metabolism, we hypothesized that MI plays a major role in regulating avian body mass and mitochondrial fuel utilization. We tested this hypothesis using integrative organismal, genomic, and cellular approaches.

RESULTS

Chronic exposure to MI prevents fat accumulation in hummingbirds

Hovering and nectar-feeding birds sustain among the highest mass-specific aerobic metabolic rates among vertebrates (*21*). Hummingbirds also rely on daily accumulated fat stores through the night (*22*). To test whether chronic exposure to 5% MI significantly altered hummingbirds' body mass regulation, we supplemented hummingbird diets with 5% MI–enhanced nectar and monitored their body mass using a perch mounted on a balance scale, which continuously recorded body mass for a perched individual. MI significantly affected evening body mass ($F_{1,6} = 12.862$, $P = 0.012$), and birds supplemented with 5% MI were lighter on average (Fig. 1A). Initial body mass significantly affected the evening body mass ($F_{1,6} = 9.45$, $P = 0.02$), and there was also a significant interaction effect between initial body mass and day ($F_{1,6} = 6.79$, $P = 0.04$). Chronic exposure to 5% MI marginally influenced morning body mass ($F_{1,6} = 5.269$, $P = 0.06$), and birds supplemented with 5% MI were lighter on average (Fig. 1B). The interaction effect between initial body mass and day was also significant ($F_{1,6} = 5.27$, $P = 0.05$). Within subjects, day number, and the interaction between initial body mass and day significantly influenced

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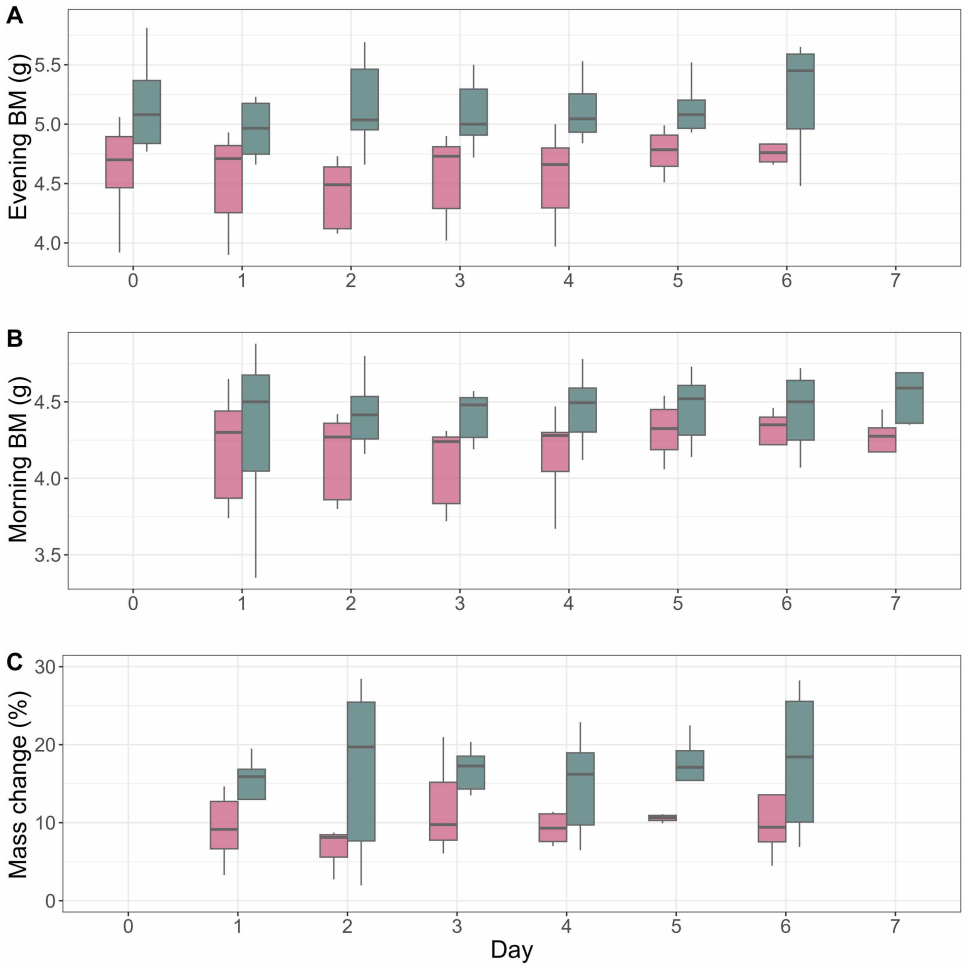

Fig. 1. Effects of chronic exposure to 5% MI (overall *N* = 13). (**A**) Average evening body mass derived from the last 10 min of an experimental day and (**B**) initial morning body mass for control birds and birds supplemented with 5% MI. The experiment started at 9 a.m. on the first day (i.e., day 0, with no morning mass measurement) and ended at 12 p.m. on day 7 (with no evening mass measurement). (**C**) Daily change of body mass (%) for each full experimental day.

morning body mass ($F_{1,63} = 5.16$, $P = 0.03$ and $F_{1,63} = 5.10$, $P = 0.03$, respectively), whereas the interaction effect between treatment and day number was marginally nonsignificant ($F_{1,63} = 3.111$, $P = 0.083$). Overall, hummingbirds supplemented with 5% MI accumulated less evening fat and maintained lower body mass during the day.

Hummingbirds can discriminate and seasonally regulate MI consumption

Hummingbird discrimination of nectar properties may be achieved via taste receptors (*23*), postingestive physiological feedback (*24*), or by a combination of these mechanisms. We designed a repeated choice experiment in which individuals were presented with two equidistant feeders containing either control nectar or nectar supplemented with different MI concentrations (either 1, 3, or 5%; table S1). In addition, as some species of hummingbirds can seasonally regulate their body mass in accordance with demands of migration (*22*, *25*, *26*), we assessed the MI consumption seasonally: postmigration, a sedentary season, and premigration.

Ingested MI, which represents the weighted mean of the MI proportion in the consumed nectar, significantly and negatively affected ensuing changes in body mass ($F_{1,184} = 5.768$, $P = 0.017$). The total volume of nectar consumed by the hummingbirds also significantly affected changes in body mass ($F_{1,184} = 19.392$, $P < 0.001$); the interaction between those two variables was nonsignificant ($F_{1,184} = 1.342$, $P = 0.248$). Seasonality did not affect the change in body mass ($F_{2,184} = 1.266$, $P = 0.284$) but did affect *ingested MI* ($F_{2,21} = 10.330$, $P < 0.001$), with *ingested MI* being higher during the postmigration season and lower during premigration (Fig. 2A). Seasonality also significantly influenced body mass at capture ($F_{2,21} = 3.854$, $P = 0.03$; Fig. 2B), which was higher during the postmigration season (Fig. 2B).

The effect of MI ingestion on hummingbird body mass is immediate, causing a decrease in body mass over a period of only 2 hours. Greater rates of overall nectar consumption were significantly correlated with higher body mass gain. However, the dynamic interplay between nectar consumption and ingested MI cannot be completely disentangled. Increased consumption of control nectar will necessarily increase body mass, but an increase of MI-enhanced nectar will have two opposing effects, as MI ingestion counteracts the body mass increase otherwise associated with ingestion of control nectar.

We also found a significant effect of seasonality on the birds' choice ($F_{2,3} = 60.392$, $P = 0.004$; Fig. 2C). During postmigration season, birds did not show preference toward the control solution, suggesting that

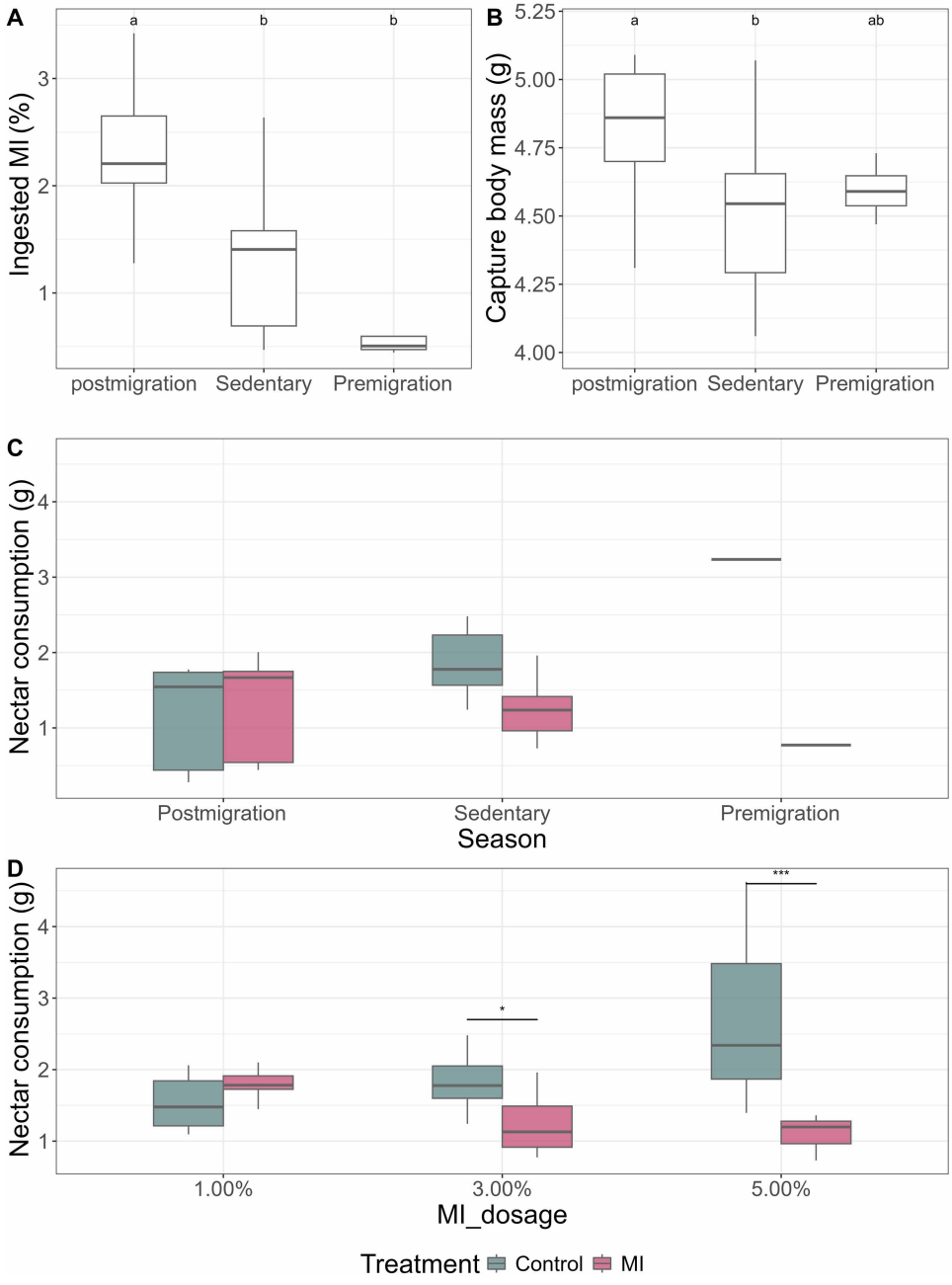

Fig. 2. Results of 2-hour exposure to MI (overall $N = 30$). (**A**) Differences in ingested MI (%) (derived from Eq. 1) across seasons. (**B**) Differences in body mass upon capture across seasons. (**C**) Differential nectar consumption in a dual choice experiment between control solution and behaviorally distinguishable MI-enhanced solution (3 and 5%) across different seasons. (**D**) Differential nectar consumption in a dual choice experiment between control solution and different dosages of MI, excluding the postmigration season. Asterisks indicate significance levels (*$P < 0.05$, ***$P < 0.001$).

postmigration hummingbirds (mostly arriving with higher body mass) consumed either low amounts of nectar or high amounts of MI. That is, heavier individuals in this experiment may deliberately consume more MI so as to regulate their body mass and reduce fat content, given that the ability of birds in general to regulate body mass is well documented (*27*–*29*). Furthermore, excluding this season disclosed that birds generally avoid higher MI concentrations ($F_{2,8} = 5.754$, $P = 0.028$; Fig. 2D). Interactions between treatment and body mass significantly affected food choice ($F_{2,42} = 4.387$, $P = 0.019$; see also table S3). These results indicate that the effect of MI ingestion on body mass is immediate, causing a decrease in body mass over a period of only 2 hours but also show that hummingbirds can detect and seasonally regulate MI consumption according to their seasonal and behavioral requirements. The seasonal movements and body mass of Anna's hummingbirds in northern California are only partially understood, but migrating populations typically arrive in fall (i.e., September to November) (*30*, *31*). It is unknown whether the urban population studied here is migratory, but we observed a

decline in bird abundance in late May, with juveniles and females returning in late August, followed by males, and all categories arriving with increased body mass postmigration (Fig. 2B). It is unclear why these hummingbirds arrive with an increased body mass; this effect may derive from a bias introduced by the timing of sampling before and after migration. However, similar phenologies have been documented for birds and relate mostly to breeding superiority (*32*) and to reduced migratory range (also known as short-stopping), suggesting that individuals could arrive with greater fat reserves as a result of shortening their overall migration trajectory due to changes in climatic conditions (*33*). Nevertheless, whereas the body masses documented in this experiment may not accurately reflect natural migratory phenology, our results reveal a consistent pattern of body mass–dependent response to MI.

MI transmembrane transporters are conserved across birds

Three main transmembrane transporters are responsible for moving MI across the cell membrane (*34*). We evaluated whether these transporters are conserved across bird species using the ConSurf algorithm (*35*). We used two additional proteins as references: CYCS, which served as a well-conserved reference sequence of cytochrome c (*36*), and B2M, which served as a reference sequence for the rapidly evolving beta-2-microglobulin (*37*). Overall, the MI-specific HMIT (H^+ myo-inositol transporter, *SLC2A13* gene) was highly conserved among analyzed species (Table 1). Similarly, the nonexclusive MI transporters SMIT1 (*SLC5A3* gene) and SMIT2 (*SLC5A11* gene) were also both highly conserved, showing similar results to those of the well-conserved CYCS. Moreover, there was no evidence for positive selection on the tested foreground branch leading to hummingbirds for any of these three MI transporters (Table 1) compared to the background of other bird species, indicating that all three transporters are unlikely to have undergone functional changes among birds. These analyses use transporters sequenced from 13 previously unsequenced species of hummingbird, which were not publicly available before this study.

The gene *SLC2A13* (HMIT) has been reported to be positively selected in the hummingbird lineage (*19*), but our analysis with a much larger dataset (including additional hummingbird species) did not support this finding. The prior evidence for selection in *SLC2A13* was based on three amino acids that appeared to be unique to the hummingbirds. However, our expanded dataset showed that these derived amino acids are not fixed within hummingbirds and are also found in other bird species not represented in the dataset of Osipova *et al.* (*19*). Variability of these amino acids in our expanded avian dataset explains why our tests found no evidence for selection on *SLC2A13*. Notably, two of those amino acids are located in close proximity (i.e., positions 248 and 250), separated by only one amino acid, and hummingbird taxa were the only ones to exhibit both amino acid mutations (Fig 1S and the Supplementary Materials). Although nectarivore species often exhibit unique regulation of or positive selection on sugar metabolism–related genes (*38*, *39*), there was no evidence of differential rates of evolution in any of the MI transporters for the stem branch leading to hummingbirds, although other algorithms [e.g., (*40*)] might suggest a different perspective. The function of MI transporters, and the role of MI in avian metabolism more generally, is likely conserved among birds, and species-specific phenotypes might be a result of regulatory changes of MI genes and not directly in their functional structure.

MI enhances the use of fats as mitochondrial fuel through affecting the PDH activity

To explore the molecular mechanism underlying fat accumulation inhibition, we measured changes in real-time respiration rates in avian QT6 fibroblasts (Japanese quail muscle fibroblasts) treated with MI using extracellular flux assays. We have verified using polymerase chain reaction (PCR) that the QT6 cells express all three transporters (fig. S2). MI treatment suppressed cellular respiration (Fig. 3A). Specifically, MI decreased basal respiration ($F_{1,4} = 16.35$, $P = 0.01$) and maximal respiration ($F_{1,4} = 7.00$, $P = 0.05$), but not proton leak ($F_{1,4} = 2.78$, $P = 0.17$) or reserve capacity ($F_{1,4} = 0.23$, $P = 0.635$; fig. 3C). Consistently, MI treatment decreased pyruvate dehydrogenase (PDH) activity ($t_2 = 17.96$, $P < 0.001$; Fig. 3B), although this reaction is substrate limited and might be a result of substrate depletion. Our results may imply that, after 2 hours of MI treatment, the media are substrate limited, suggesting that the cells supplemented with MI might recover by supplementing the media with FAs. When repeating the extracellular flux assay while supplementing the cells with palmitate (Fig. 4A), cells increased use of FAs as mitochondrial fuel in response to MI. Furthermore, cells treated with MI and supplemented with FA showed no differences from control cells (Fig. 4A). Palmitate supplementation alone did not alter maximal respiration. However, when coupled with MI treatment, palmitate significantly increased maximal respiration ($F_{3,6} = 7.33$, $P = 0.02$; Fig. 4B), revealing a significantly higher external FA oxidation for MI-treated cells ($t_3 = -3.42$, $P = 0.042$; Fig. 4C).

DISCUSSION

In this study, we elucidate the importance of MI for avian metabolism and body mass regulation. Hummingbirds supplemented with MI accumulated less evening fat and maintained lower body mass during the day. These body mass differences were smaller in the

Table 1. Conservation score (i.e., geometric mean for all positions), number of amino acids, and the conservation status of each protein. Data were analyzed using the ConSurf algorithm.

Transporter	Conservation score	Amino acids	Conservation status
SLC2A13 (HMIT)	7.55	646	Conserved
SLC5A11 (SMIT1)	7.51	678	Conserved
SLC5A3 (SMIT2)	7.94	713	Conserved
CYCS	7.3	315	Conserved
B2M	5.03	357	Variable

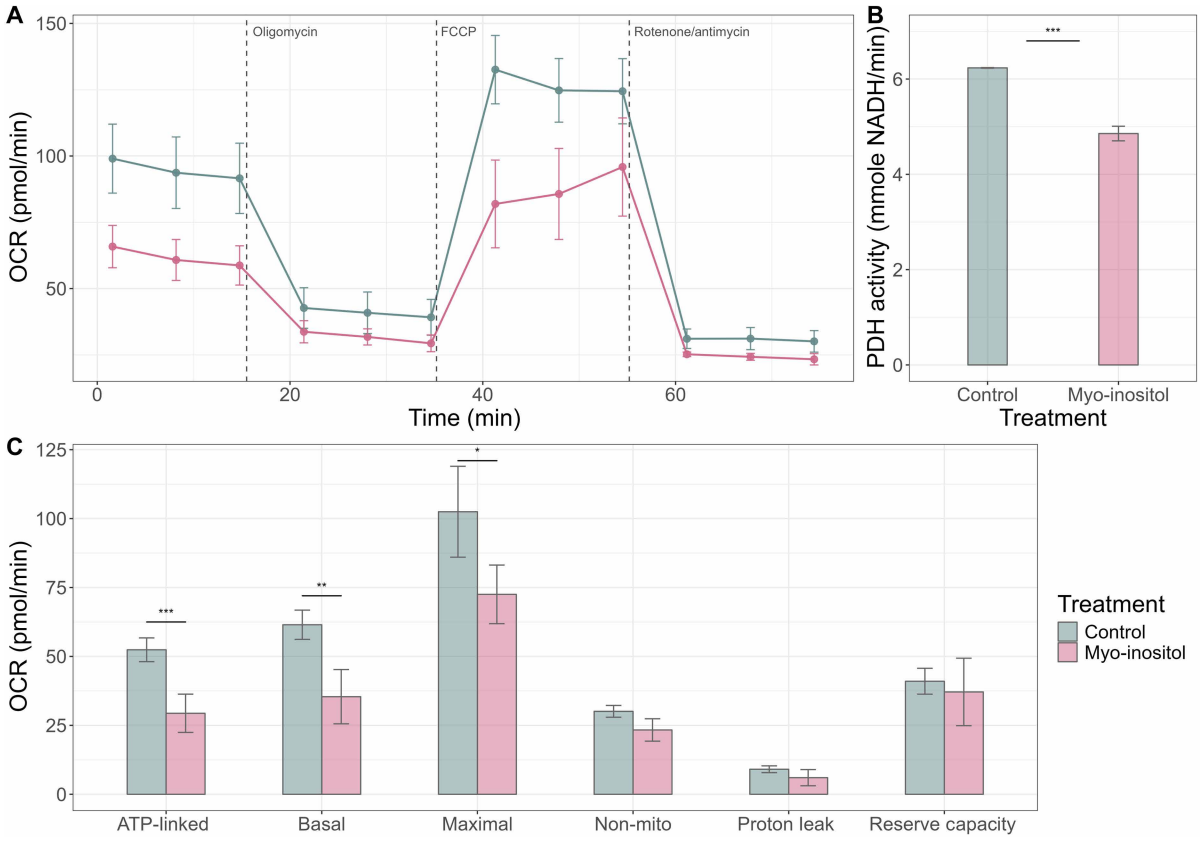

Fig. 3. Changes in cellular bioenergetics in response to 5% MI treatment. (**A**) OCR in QT6 cells with and without 5% MI and their corresponding (**B**) PDH activity (mmol NADH/min), and (**C**) mitochondrial function. All results are normalized to total protein. Asterisks indicate significance levels (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$).

morning as the birds use stored fats during the night. Similar results have been previously reported in both human and model organisms, for which MI supplementation decreased body mass index and improved diabetic symptoms (*41*, *42*). MI is not commonly found in nectar or fruits (*10*), but elevated MI plasma concentrations have been recorded in some avian species, captured upon arrival at a stopover site (*18*). During recovery from fasting and endurance migratory flight, birds primarily rely on lipids for energy, gradually increasing their food intake over the first few days at the stopover (*43*). It is possible that birds are capable of internally regulating their mitochondrial fuel source and may regulate body mass by enhancing FA oxidation, using both endogenous and exogenous MI. Such regulation can vary across seasons as a consequence of matching fuel source relative to the demands of migration. MI concentrations in these experiments were much higher than those found in natural food sources such as nectar. We do not expect hummingbirds to differentiate among nectars by their MI concentration, and our results do not necessarily indicate dietary selection of hummingbirds in the wild. Experimental use of these concentrations was instead designed to elucidate, via the ingestion of MI, any associated regulation of body mass.

Mechanistically, the fuel regulation in the mitochondria may derive from interaction of MI with the PDH complex, which is known to regulate the Krebs cycle and the main enzyme linking different components of energy production (*44*), which was suppressed by MI treatment in our experiments. Different complexes of PDH are up-regulated in muscle and liver tissues of hummingbirds (*19*), and the PDH complex in mammalian adipocytes is known to be activated by inositol 3 phosphate (*45*), of which MI is the precursor. MI also inhibits mitochondrial fission by repressing AMPK activation in mammalian cells (*46*), thus highlighting that MI could affect mitochondrial function in multiple ways, including the modulation of the PDH complex and the regulation of mitochondrial dynamics. Birds have various mechanisms to up-regulate lipid utilization, especially during migratory flight (*2*, *43*, *47*), which is fueled almost exclusively by lipids (*48*). FAs provide more adenosine 5′-triphosphate (ATP) per mole than carbohydrates and have a lower water content, thus optimizing physiological needs during migration. High MI levels have been recorded in birds recovering from fasting (*18*), which, alongside our results, suggests an additional mechanism enhancing FA oxidation by birds.

Our results indicate that the three MI transporters are highly conserved across bird species and are unlikely to have undergone functional adaptive changes among birds in general and hummingbirds in particular. MI and its derivatives may act in other taxa as secondary messengers downstream of insulin receptors (*9*), our results imply a link between avian insulin resistance and MI functions. Whereas birds are mostly insensitive to the glucose-lowering effects of insulin, this hormone does increase FA intake by targeting the FA translocase CD36 (*49*), suggesting relictual insulin sensitivity and a possible shared evolution of MI-based mass regulation and avian insulin resistance.

Overall, our study elucidates the physiological effects of MI exposure on body mass regulation in birds and identifies the underlying

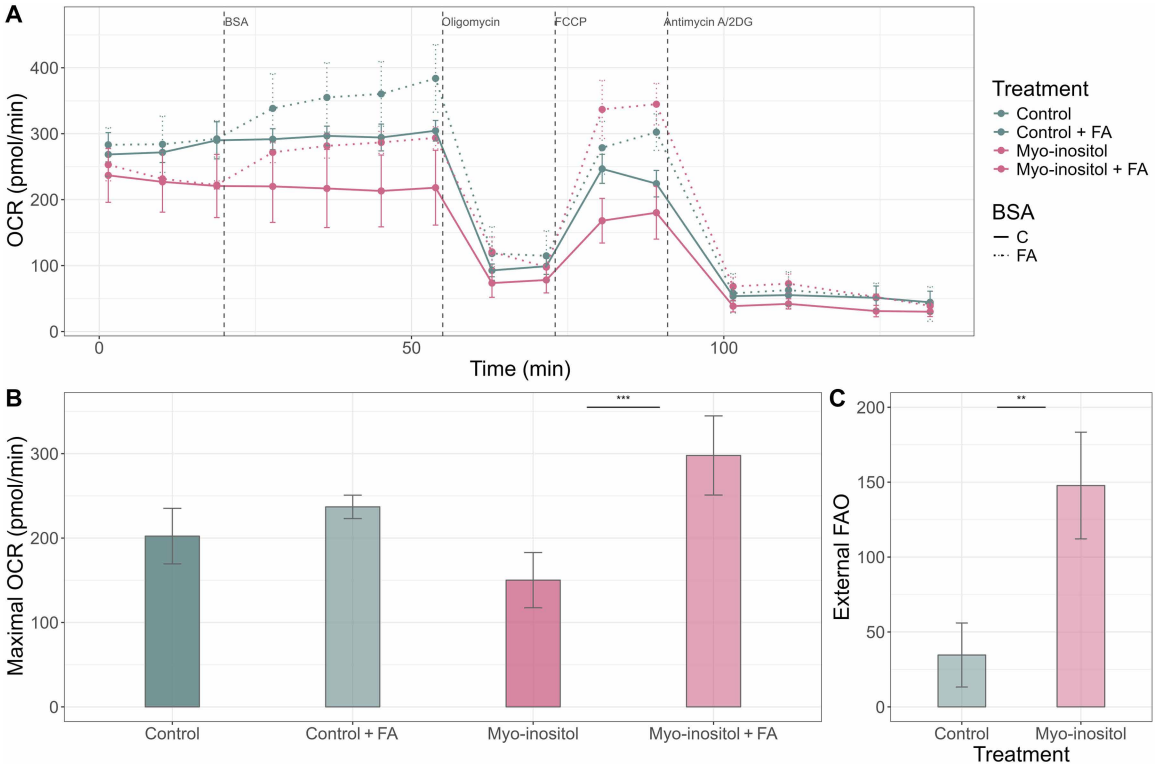

Fig. 4. Mitochondrial OCR in response to 2.5% MI exposure using FA oxidation assay. (**A**) QT6 fibroblast OCR with and without MI, supplemented with either bovine serum albumin (BSA) (solid line) or BSA + palmitate (dashed line) and (**B**) maximal respiration values. (**C**) External FA oxidation for MI and control treatment, as calculated from the differences between maximal respiration with and without palmitate. Asterisks indicate significance levels (**$P < 0.01$, ***$P < 0.001$).

cellular mechanism. MI regulates mitochondrial fuel selection by forcing cells to metabolize FAs, via an effect on PDH complexes. The dietary and behavioral modulation of MI consumption varied with season and correspondingly with individual body mass, indicating the key role of ecology relative to the physiological regulation of cellular metabolism. Nonetheless, MI concentrations in nectar and other natural foods are relatively low, and birds likely regulate MI internally. Our findings underscore the importance of MI in avian metabolism, particularly in the context of hyperglycemia and fat accumulation, offering insights into the evolutionary adaptations of birds for energy management in the context of insulin resistance.

MATERIALS AND METHODS

At the organismal level, Anna's hummingbirds (*Calypte anna*) were used to assess the role of dietary MI in body mass regulation. At the genomic level, we used nucleotide multiple sequence alignments for the three main MI transporters (*SLC2A13*, *SLC5A3*, and *SLC5A11*) to study potential protein conservation among diverse bird species and to assess whether these genes were positively selected and/or uniquely mutated in the hummingbird lineage. Last, we used QT6 cells (muscle fibroblast cell line from Japanese Quails, *Coturnix japonica*) to experimentally assess the effects of MI on avian mitochondrial fuel utilization.

Organismal studies

Chronic MI exposure

Adult male hummingbirds (*C. anna, N* = 13) were captured at the University of California, Berkeley (under IACUC permits protocol). Birds were individually housed in 1-m^3 mesh cubes maintained at constant air temperature and humidity and on a 12:12-hour, 6 a.m. to 6 p.m. circadian rhythm, with completely dark nights. The birds were provided with ad libitum artificial nectar (Nektar Plus, Nekton USA Inc.), made daily at approximately 20% v/v in deionized water. After one full day of acclimation, the birds were provisioned over 7 days with either ad libitum control nectar (as during acclimation) or with ad libitum experimental nectar [20% Nektar Plus, mixed with 5% powdered MI (Sigma-Aldrich)]. Each cage contained one perch mounted on a balance scale (FX-1200i, A&D), which continuously recorded body mass for a perched bird. Morning body mass was derived from one initial body mass measurement taken directly after lights were turned on, whereas evening body mass was derived from an average of masses recorded during the final 10 min before darkness so as to incorporate variable fluid elimination postfeeding.

Dietary preference (short-term MI exposure)

Experiments occurred from morning to early afternoon (9 a.m. to 2 p.m.) from November 2022 to February 2024 (table S1). For any given trial, an individual was placed in a Plexiglas cube (0.9 m by 0.9 m by 0.9 m) containing a perch and two equidistant 10-ml syringe feeders with either control nectar (as described above) or nectar supplemented with different MI concentrations (either 1, 3, or 5%; table S1).

After 1 hour of a 2-hour experimental trial, syringe locations were switched to avoid location bias. Each bird underwent 7 days of repeated trials such that the first three trials were assumed to represent a learning period in the experiment. Birds exhibiting location bias (i.e., feeding at only one location throughout two trials) were removed

from the analysis ($n = 2$). For each trial, we determined the mass of nectar removed from each syringe, along with body mass before and after the trial. All organismal studies were performed under the auspices of US Fish & Wildlife Service (MB054440) and State of California Scientific Research (SC-6627) permits, and an IACUC protocol from the University of California, Berkeley.

Genetic sequence analysis

Nucleotide sequences of the three MI transporters (*SLC2A13*, *SLC5A11*, and *SLC5A3*, coding for HMIT, SMIT1 and SMIT2, respectively) were obtained using National Center for Biotechnology Information (NCBI) orthologs (*50*). In addition to sequences obtained through NCBI, we included sequences of 13 hummingbird species, obtained as part of an independent project to study hummingbird genetic evolution and phylogenetics. Tissue samples for these hummingbirds were obtained from multiple museums (table S4), and DNA was extracted with a modified salt extraction protocol (*51*). A KAPA (Roche) library prep kit was used to make libraries, which were pooled before undergoing sequence capture with a custom SeqCAP EZ HyperCap sequence-capture kit from Nimblegen (Roche). The three MI transporter genes were targeted in the sequence-capture kit using mRNA sequences obtained from the *C. anna* genome (*52*). The captures were sequenced on two NovaSeq S4 150 PE lanes. The reads underwent quality control cleaning using HTStream v 1.3.3 (HTStream version 1.3.3. https://github.com/s4hts/HTStream n.d.) and then were mapped using the BWA-MEM 0.7.17-r1188 mapper (*53*) to a reference sequence for each gene from the *C. anna* genome. Consensus sequences for each gene for each species were obtained using ANGSD v. 0.933–79-gda26ba4 (*54*). We chose species from this project so that we would have representatives of each of the nine major clades of hummingbirds (*55*) and that the species chosen could represent the sequence variation observed in the *SLC2A13* gene at the sites that Osipova *et al.* (*19*) identified as potentially under selection.

Overall, our alignments comprised 97, 28, and 52 species for *SLC2A13*, *SLC5A11*, and *SLC5A3*, respectively. In addition, two other proteins were used as references: CYCS (comprising 114 sequences), which served as a well-conserved reference sequence of cytochrome c (*36*), and B2M, which served as a reference sequence for beta-2-microglobulin, (comprising 90 sequences), which was recently suggested to be a rapidly evolving protein (*37*). Conservation of amino acid sequence was analyzed and mapped using ConSurf (*35*), using Bayesian calculation and the HMMER homology search algorithm. We also carried out positive selection analyses using the same sequence alignments for *SLC2A13*, *SLC5A11*, and *SLC5A3*. Tests of positive selection with aBSREL (*56*) algorithm in Datamonkey (*57*, *58*) using codon alignments from PRANK v.170427 (*59*) were used for the same set of sequences for each gene. We used the branch of hummingbirds as foreground and compared it with a diverse background of bird species, testing whether there is an increase in nonsynonymous mutations in hummingbird sequences, configuring an adaptation of these genes in this species' evolution.

For *SLC2A13*, we have also generated a phylogenetic tree of those avian species used in our analyses, with the protein position of interest in the *SLC2A13* transporter (fig. S1). The tree was obtained from Vertlife (https://vertlife.org/) using the "Hackett All Species" phylogeny. The amino acids of interest were visualized on the avian phylogeny using functions from the ggtree (*60*), ape (*61*), and dplyr (*62*) R packages.

Extracellular flux assays and PDH activity

Cellular experiments were conducted using QT6 Japanese Quail (*C. japonica*) muscle fibroblasts [American Type Culture Collection (ATCC), CRL-1708] obtained from ATCC through UC Berkeley's cell culture facility. All cell culture experiments and measurements were conducted using cell passage numbers 8 to 10. Cells were maintained at 37°C with 5% CO_2, using Dulbecco's modified Eagle's medium/Nutrient Mixture F-12 (Gibco) supplemented with 5% fetal bovine serum, 1% chicken serum, 10% tryptose broth (Sigma-Aldrich), and 2% penicillin-streptomycin. To verify that the cells express all three MI transporters, we quantified the PCR products using specific primers designed to amplify *SLC2A13*, *SLC5A3*, and *SLC5A11* in the QT6 cell line (fig. S2). For this assay, we used the DNeasy Tissue Kit (QIAGEN, Hilden, Germany) and the PCR Master Mix Kit (Thermo Fisher Scientific Inc. USA). PCR products were then loaded onto an agarose gel (2%) and were separated by electrophoresis to assess the presence and size of the amplified fragments. To determine the effect of MI on oxygen consumption (OCR) and extracellular acidification rates (ECAR), we used the Mito-Stress Test kit with the XFp mini extracellular flux analyzer (Agilent Technologies; Santa Clara, CA). Following a 2-hour incubation in growth medium supplemented with 5% MI (0.2776 M), the OCR and ECAR were measured while injecting oligomycin (1 μM), carbonyl cyanide-*p*-trifluoromethoxyphenylhydrazone (FCCP, 2 μM), and rotenone/antimycin A (0.5 μM). The FCCP concentration was optimized for this cell line, with a cell count of 40,000 per well. To determine the effect of MI on FA oxidation, we used an FA oxidation assay with the XF24 extracellular flux analyzer (Agilent Technologies; Santa Clara, CA). The cells were incubated overnight in low-serum medium composed of 0.5 mM D-glucose, 1 mM L-glutamine, 0.5 mM L-carnitine, and 1% FBS. When supplementing cells with 5% MI in addition to this low-serum medium, the OCR was excessively reduced (fig. S3), and, consequently, MI supplementation was reduced to 2.5% for the FA oxidation assay. After a 2-hour incubation period in low-serum medium supplemented with 2.5% MI (0.139 M), OCR and ECAR were measured while injecting oligomycin (1 μM), FCCP (2 μM), and antimycin A/2-deoxy glucose (0.5 μM). The FCCP concentration was optimized for this cell line, with a cell count of 70,000 per well. Mitochondrial function was calculated according to Divakaruni *et al.* (*63*). Shortly thereafter, basal respiration was calculated as the OCR before inhibitors were added, less the nonmitochondrial OCR as measured using antimycin A (complex III inhibitor) and 2-deoxy glucose (glycolysis inhibitor). Proton leak, defined as the remaining basal respiration not coupled to ATP production, was calculated using the mean OCR after oligomycin injection, minus nonmitochondrial respiration. ATP-linked respiration was measured as basal respiration minus the mean OCR after oligomycin (ATP synthase inhibitor) injection. Maximal respiration was calculated as the OCR after FCCP (uncoupling agent that increases oxygen consumption by complex IV to maximum) injection, and spare capacity was calculated as the difference between maximal respiration and basal respiration. Last, we used a commercial PDH activity assay (MAK183, Sigma-Aldrich) to assess the effect of MI on PDH.

Statistical analyses

Chronic exposure

The effect of chronic exposure to MI on body mass regulation (also comparing morning and evening body masses) was assessed using a split-plot analysis of variance (ANOVA) model, with treatment as

the between-subject factor and day as a within-subject factor. Initial body mass (i.e., value on day 0) was added to the model as a covariate.

Dietary preference

To assess the effect of a 2-hour exposure to MI on hummingbird body mass, we calculated the weighted mean of *ingested MI* from the total volume of nectar consumed from both syringes and the corresponding MI content from each syringe

$$Ingested\ MI[\%] = \frac{MI\ dosage * MI\ \ consumed[g]}{Total\ food\ consumption[g]} \times 100 \quad (1)$$

To assess seasonality effects, experimental months were segregated into three seasons: premigration (April and May), postmigration (August and September), and a sedentary season (October to March). The effect of seasonality on initial body mass at capture was determined using analysis of covariance (ANCOVA) with body mass as the response variable and season as the independent factor. Similarly, the effect of ingested MI on the change in body mass over a 2-hour period of exposure was determined using an ANCOVA, with body mass change (as normalized to the initial mass) as the response variable, and ingested MI, total nectar consumption, and season as independent variables.

We further assessed effects of a 2-hour exposure to MI at different dosages (i.e., treatments) on hummingbird food choice and corresponding body mass change using a split-plot ANOVA model, with either treatment or body mass change (as normalized to initial mass) as the between-subject factor and trial number as a within-subject factor. Initial body mass at capture was added to the model as a covariate. Season was added as a factor to the initial model but was then excluded alongside the postmigration data that were collected for the final analysis (table S2).

Extracellular flux assays

To assess the effects of a 2-hour exposure to MI on avian fibroblasts, we used multivariate ANOVA, with values of basal respiration, ATP-linked respiration, proton leak, maximal respiration, reserve capacity, and nonmitochondrial respiration (as derived from OCR) as the dependent variables, and treatment as the independent factor. To assess the effects of a 2-hour exposure to MI on PDH activity, the bioenergetic respiration profile and the rate of exogenous FA oxidation were conducted using *t*-tests, comparing between control and MI experimental group. For all statistical analyses, significance level was set to $P = 0.05$, with values of $0.05 < P < 0.1$ considered marginally nonsignificant.

Supplementary Materials

The PDF file includes:

Figs. S1 to S3
Tables S1 to S4
Legends for data S1 to S3
Data S4 to S6

Other Supplementary Material for this manuscript includes the following:

Data S1 to S3

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