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Author for correspondence:
Paulo H. C. Mesquita
e-mails: paulo-mesquita@omrf.org; pmesquita@live.com

†Present address: Aging & Metabolism Research Program, Oklahoma Medical Research Foundation 825 NE 13th St., Office W121, Oklahoma, OK, 73104, USA.

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Mitochondrial remodelling supports migration in white-crowned sparrows (*Zonotrichia leucophrys*)

Paulo H. C. Mesquita[1,3,†], Emma M. Rhodes[2], Kang Nian Yap[2,4], Breanna J. Mueller[1], Geoffrey E. Hill[2], Wendy R. Hood[2] and Andreas N. Kavazis[1]

[1]School of Kinesiology, and [2]Department of Biological Sciences, Auburn University, Auburn, AL 36849, USA
[3]Aging & Metabolism Research Program, Oklahoma Medical Research Foundation, Oklahoma City, OK, USA
[4]Department of Biology, Norwegian University of Science and Technology, Trondheim 7491, Norway

PHCM, 0000-0003-1112-5493; EMR, 0000-0003-4546-7838; GEH, 0000-0001-8864-6495; WRH, 0000-0003-4546-7838

The migratory movements undertaken by birds are among the most energetically demanding behaviours observed in nature. Mitochondria are the source of aerobic energy production on which migration depends, but a key component of mitochondrial function, mitochondrial remodelling, has not been investigated in the context of bird migration. We measured markers of mitochondrial remodelling in the skeletal muscles of the Gambel's (migratory) and Nuttall's (non-migratory) white-crowned sparrows within and outside migratory periods. Gambel's were collected in (i) a non-migration period (baseline), (ii) preparation to depart for spring migration (pre-migration) and (iii) active autumn migration (mid-migration). Nuttall's were collected at timepoints corresponding to baseline and mid-migration in Gambel's. Across all sampling periods, we found that migratory birds had greater mitochondrial remodelling compared with non-migratory birds. Furthermore, birds from the migratory population also displayed flexibility, increasing several markers of mitochondrial remodelling (e.g. NRF1, OPA1 and Drp1) pre- and during migration. Further, the greater levels of mitochondrial remodelling and its upregulation during migration were specific to the pectoralis muscle used in flapping flight. Our study is the first to show that mitochondrial remodelling supports migration in Gambel's white-crowned sparrows, indicating a highly specific and efficient phenotype supporting the increased energetic demands of migration.

1. Background

Long-distance migration is one of the most energetically demanding activities documented in animals. In birds, migratory movements can vary from short-distance movements that cover hundreds of miles to voyages that span the globe, as is the case in the bar-tailed godwit (*Limosa lapponica*) that travels over 10 000 km with each migratory event [1]. To support the energetic challenges of long-distance flights, birds experience an array of physiological adaptations spanning from whole organism to subcellular processes that persist throughout the year [2,3] or are upregulated to support migratory movements and then are downregulated when migration has ended [4–6].

One obvious challenge faced by birds during migration is the increased demand for energy in the form of adenosine triphosphate (ATP). In preparation for migration, birds increase their food intake, the size of flight muscles and the amount of fat stored to fuel migration [7–10]. In addition, migratory birds have an enhanced delivery of both oxygen [2,11] and fuel to muscles during migration [12,13]. As the organelles responsible for aerobic

energy production, mitochondria use these resources to produce the ATP needed to fuel migratory movements [14–16]. It has recently been shown that migratory Gambel's white-crowned sparrows (*Zonotrichia leucophrys gambelii*) support the energy demands of migration by maintaining a higher volume of mitochondria in their pectoralis muscle than non-migratory Nuttall's white-crowned sparrows (*Zonotrichia leucophrys nuttalli*). Further, the migratory sparrows upregulate their capacity for oxidative phosphorylation, displaying higher maximal and basal respiratory function just prior to and during migration, but then downregulate their oxidative phosphorylation when migration has ended [6]. The question remains: what are the specific mechanisms that facilitate this increase in oxygen utilization efficiency and rate of ATP production?

A rarely considered aspect of respiratory capacity in comparative studies is the process responsible for remodelling the mitochondrial network. Mitochondria form complex and highly adaptable networks that undergo constant remodelling to maintain and, potentially, increase the capacity of a cell to produce ATP [17,18]. These processes are particularly notable in skeletal muscle, where mitochondria form complex, three-dimensional reticula [19]. Remodelling is achieved via processes that are cumulatively referred to as mitochondrial dynamics; these include mitochondrial biogenesis, which can increase the number and, ultimately, the volume of mitochondria in the tissue; mitophagy, which removes dysfunctional mitochondria; and mitochondrial fusion and fission [20]. During mitochondrial fusion, adjacent mitochondria fuse their outer and inner membranes to create a larger organelle, which allows for membrane potential conduction between fused mitochondria and minimizes metabolite diffusion [19]. In contrast, mitochondrial fission results in the division of mitochondria in two, allowing mitochondria to remove damaged portions while retaining a functional core [21]. Thus, proper mitochondrial remodelling can contribute to changes in the morphological characteristics of the mitochondrial network and improve the rate of oxygen utilization, allowing for greater ATP production [20,22].

Mitochondrial remodelling has been shown to change in response to different conditions (i.e. exercise and aging) in both humans [23–25] and rodents [26–28]. In particular, markers of mitochondrial fusion and fission have been shown to increase in skeletal muscle in response to high-intensity interval training, which was associated with improved mitochondrial respiratory capacity [29]. When these processes fail, an individual's capacity for oxidative phosphorylation is hindered [17,30]. Our understanding of how free-ranging animals adapt to life cycle stages that require changes in mitochondrial respiratory capacity, as observed in the white-crowned sparrows, remains limited [15]. Changes in mitochondrial remodelling processes are one possible suite of mechanisms that would enable animals to increase energy output for activities such as migration, but mitochondrial dynamics has never been quantified in animals living in natural environments.

While there are a multitude of studies evaluating mitochondrial dynamics in humans, mice and other model species, there is no prior work on wild birds. This distinction is important because wild birds are subject to challenges requiring modifications of ATP production that are beyond the ranges of change demanded of model organisms [31], and mitochondrial fission and fusion dynamics are likely to play a critical role in the regulation of mitochondrial respiration in the diversity of energetic challenges that a wild bird faces throughout its life. So far, the only measurements of mitochondrial dynamics in birds have been limited to one study in domestic pigeons and several studies in poultry [32–34], although mitochondrial morphology has been evaluated in a hummingbird [35]. In pigeons (*Columba livia*), the expression of genes involved in mitochondrial biogenesis and fusion has been found to be higher in the skeletal muscle during the winter compared with summer, an adaptation that is likely to support nonshivering thermogenesis [36].

Given that mitochondrial remodelling appears to play an important role in improving mitochondrial respiratory function with exercise in humans and laboratory animals, we hypothesize that these processes will also play important roles in supporting an upregulation of mitochondrial respiratory function in birds during migration. The aim of the current study is to evaluate variation in markers of mitochondrial remodelling in the skeletal muscles of migratory and non-migratory populations of a songbird species as a first step in determining whether altered mitochondrial dynamics underlies changes in mitochondrial respiratory capacity of skeletal muscle in birds during migration. We selected white-crowned sparrows for this work because some populations of this species are sedentary with no seasonal movement, while other populations undertake long-distance migrations between separate wintering and breeding areas.

We focused on Gambel's and Nuttall's white-crowned sparrows because it has previously been demonstrated that they have underlying differences in mitochondrial respiratory capacity [6], they contain differing mitochondrial genotypes and they share a recent common ancestor with similar life cycle stages, with the exception of migration status [11,37]. We sampled birds in both migratory and non-migratory periods to determine whether changes in mitochondrial remodelling reflect fixed differences between migratory and non-migratory subspecies or if they are the result of individual flexibility within migrants. Further, to determine whether changes are global (whole-body) or specific to muscles actively recruited during flight, we measured mitochondrial remodelling in both a wing muscle that creates flapping movement and a leg muscle that plays no active role in flight. To this end, we assessed the pectoralis muscle, which lifts the bird during the energy-demanding downstroke of the wing [38], as well as in the quadriceps muscle, which plays no active role in flapping flight. Given a previous work on mitochondrial respiratory function [6], we predicted that mitochondrial remodelling would be upregulated in a flexible manner, such that Gambel's migrants would have similar mitochondrial remodelling compared with Nuttall's non-migrants at baseline but that they would upregulate mitochondrial remodelling before and during migration. In addition, we predicted that the upregulation of mitochondrial remodelling in migrants would be specific to the flight muscle. Finally, we predicted that the relative expression of most mitochondria dynamics markers would correlate with mitochondrial respiratory function in the migrants, providing an indication that these processes could contribute to the observed differences in oxidative phosphorylation between migrants and non-migrants.

2. Material and methods

(a) Ethical approval

The current study was approved by the Institutional Animal Care and Use Committee (IACUC) at Auburn University (PRN #2019–3549). Birds were collected using Migratory Bird Permit #MB49547D and California state permit #210190009–21019–001.

(b) Experimental design and data collection

A complete description of the study design and sampling area has been previously published [6]. In short, two subspecies of the white-crowned sparrow (*Zonotrichia leucophrys*) were collected: *Z. l. nuttalli* (Nuttall's, non-migratory) and *Z. l. gambelii* (Gambel's, migratory). Birds were collected throughout the day using baited traps and mist nets at three locations in California: (i) Davis, Yolo County (38.527943,−121.79061), temperate, dry/hot summer climate; (ii) Inyo National Forest (37.917256,−119.254190), cold/temperate dry/warm to hot summer climate; (iii) Marin Headlands (37.832165,−122.538683), temperate dry/warm summer. Climate types were determined according to Beck *et al*. [39]. Gambel's individuals were collected at three timepoints: (i) December, after completion of autumn migration and before the beginning of spring migration (baseline; n = 15, 3 females, 12 males); (ii) April, immediately prior to departure for and during preparation for migration (pre-migration; n = 15, 2 females, 13 males); (iii) September, when birds are actively migrating (mid-migration; n = 15, 9 females, 6 males). Nuttall's individuals were collected during the baseline (n = 15; 3 females, 12 males) and mid-migration (n = 15; 2 females, 10 males, 3 non-identified) timeperiods to coincide with Gambel's collections. We did not collect Nuttall's individuals during a period equivalent to the pre-migration period of Gambel's sparrows because this is a period when Nuttall's are breeding. Owing to logistical constraints and bird availability, we were unable to collect equal numbers of females and males, although Rhodes *et al*. [6] showed that the impact of sex on mitochondrial respiration was minimal. Once birds were captured, morphological parameters were determined, including fat and muscle scores, wing chord (mm), tarsometatarsus length (mm) and mass (g) as previously described [6]. Birds were euthanized via decapitation in accordance with PRN #2019–3549, and the right pectoralis and quadriceps muscles were excised, flash-frozen in liquid nitrogen and stored at −80°C for future assays.

(c) Western blots

Approximately 25–50 mg of each pectoralis and quadriceps muscles were homogenized in cell lysis buffer (Cell Signaling) using tight-fitting plastic pestles. Protein concentrations were determined using a bicinchoninic acid (BCA) assay (Thermo Fisher Scientific). Samples were prepared for Western blotting at 1 µg µl^{-1}, 10 µl loaded onto a 4%–15% sodium dodecyl sulfate (SDS)-polyacrylamide gel and transferred to preactivated polyvinylidene difluoride membranes. Membranes were stained for Ponceau S, blocked for 1 h with 5% nonfat milk powder and incubated overnight at 4°C with the following primary antibodies at 1 : 2000 dilution: total OXPHOS rodent (Abcam, Cambridge, MA, USA; Cat. no. ab110413, RRID:AB_2629281), peroxisome proliferator-activated receptor-γ coactivator 1α (PGC-1α, GeneTex Irvine, CA, USA; Cat. no. GTX37356, RRID:AB_11175466), nuclear respiratory factor 1 (NRF1; GeneTex; Cat. no. GTX103179, RRID:AB_11168915), mitochndrial transcription factor A (TFAM; Abnova Corporation, Taipei City, Taiwan; Cat. no. H00007019-D01P, RRID:AB_1715621), PTEN-induced putative kinase 1 (PINK1, Cell Signaling Technology; Danvers, MA, USA; Cat. no. 6946, RRID:AB_11179069), Parkin (Cell Signaling Technology; Cat. no. 2132, RRID:AB_10693040), Mitofusin 1 (MfN1, Cell Signaling Technology; Cat. no. 14739, RRID:AB_2744531), Mitofusin 2 (MfN2, BioVision, Milpita, CA, USA; Cat. no. 3882–100, RRID:AB_2142625), Optic Atrophy 1 (Opa1, Cell Signaling Technology; Cat. no. 67589, RRID:AB_2799728), Fission protein 1 (Fis1, Abcam Cat. no. ab71498, RRID:AB_1271360) and Dynamin-related protein 1 (DRP1, Novus, Centennial, CO, USA; Cat. no. NB110–55288SS, RRID:AB_921147). Following primary antibody incubations, membranes were washed three times in Tris-buffered saline with tween-20 (TBS-T) for 5 min and incubated for 1 h with horseradish peroxidase-conjugated anti-rabbit IgG (Cell Signaling Technology; Cat. no. 7074, RRID:AB_2099233) or anti-mouse IgG (Cell Signaling Technology Cat. no. 7076, RRID:AB_330924). Membranes were then washed in TBS-T, developed using chemiluminescent substrate (Millipore, Burlington, MA, USA) and digitally imaged. Raw target band densities were obtained and normalized by Ponceau densitometry values.

(d) Data analysis

Statistics were performed using RStudio v. 2022.12.0 [40]. Differences in the dependent variables were analysed using two-way ANOVA tests [group (G= migratory, non-migratory) × time (T, baseline, pre-migration, mid-migration)], followed by Tukey *post hoc* tests when appropriate. Statistical significance was established at $\alpha < 0.05$. All data are expressed as mean ± s.d. and 95% confidence intervals (CIs) are presented for statistically significant differences. Because of problems experienced during sample processing, one quadriceps sample from a Nuttall's non-migratory during mid-migration individual was lost, resulting in a sample size of 14. The sample size for both groups and muscles at other timepoints was 15. Furthermore, we tested the association between the markers of mitochondrial remodelling in the pectoralis muscles investigated in the current study with values of mitochondrial respiratory function in the pectoralis muscles reported in Rhodes *et al*. [6] using separate linear models of the migratory and non-migratory subspecies, with adjusted R^2 reported.

3. Results

(a) Morphology parameters

Migratory white-crowned sparrows had a greater muscle score compared with non-migratory white-crowned sparrows (0.43 ± 95% CI [0.29]). There was a main effect of time, such that muscle scores were greater at pre-migration compared with baseline (0.88 ± 95% CI [0.46]) and with mid-migration (1.12 ± 95% CI [0.46]). A significant G × T interaction was found for muscle scores, in which migratory white-crowned sparrows had greater muscle scores at pre-migration compared with baseline (1.33 ± 95% CI [0.66]) and mid-migration (1.07 ± 95% CI [0.66]). Migratory white-crowned sparrows also had greater muscle scores at pre-migration compared with non-migratory white-crowned sparrows at baseline (0.87 ± 95% CI [0.66]) and mid-migration (1.60 ± 95% CI [0.66]). Further, non-migratory white-crowned sparrows had greater muscle scores at baseline than at mid-migration (0.73 ± 95% CI [0.66]).

For fat scores, there was no significant main effect of group ($p = 0.082$) but a significant effect of time ($p < 0.001$), such that fat scores were greater at pre-migration compared with baseline (0.88 ± 95% CI [0.46]) and mid-migration (1.12 ± 95% CI [0.46]). A significant G × T interaction was found for fat scores, in which migratory white-crowned sparrows had greater fat scores at pre-migration compared with baseline (1.00 ± 95% CI [0.63]) and mid-migration (0.67 ± 95% CI [0.63]). Migratory white-crowned sparrows also had greater fat scores pre-migration compared with non-migratory white-crowned sparrows mid-migration (1.20 ± 95% CI [0.63]). Further, non-migratory white-crowned sparrows had greater fat scores at baseline compared with mid-migration (0.80 ± 95% CI [0.63]). Detailed muscle and fat scores data and descriptive statistics can be found in electronic supplementary material, tables S1 and S2. Importantly, morphology data presented here differ from those reported by Rhodes *et al*. [6] because only a subset of the sample was used in the current study.

(b) Mitochondrial complexes

In the pectoralis muscle, there was a significant main effect of group for complexes I, II and IV, in which the migratory white-crowned sparrows had greater protein levels compared with non-migratory white-crowned sparrows (Complex I: 0.93, ±95% CI [0.35], $p < 0.001$; Complex II: 1.29, ±95% CI [0.59], $p < 0.001$; Complex IV: 2.73, ±95% CI [0.97]). A significant G × T interaction was found for CI, in which migratory white-crowned sparrows had greater CI protein content at pre-migration compared with non-migratory white-crowned sparrows at baseline (1.00, ±95% CI [0.80], $p = 0.006$) and at mid-migration (1.08, ±95% CI [0.35], $p = 0.002$). Migratory white-crowned sparrows also had greater CI protein content at mid-migration compared with baseline (0.92, ±95% CI [0.81], $p = 0.017$) and compared with non-migratory white-crowned sparrows at baseline (1.31, ±95% CI [0.81], $p < 0.001$) and mid-migration (1.38, ±95% CI [0.81], $p < 0.001$). Complex II protein content was greater in the pectoralis muscle of migratory white-crowned sparrows at pre-migration compared with non-migratory counterparts at mid-migration (1.56, ±95% CI [1.33], $p = 0.012$) and greater in migratory white-crowned sparrows mid-migration compared with non-migratory white-crowned sparrows at baseline (1.63, ±95% CI [1.35], $p = 0.009$) and mid-migration (1.95, ±95% CI [1.35], $p < 0.001$). There was no significant main effect of time for any of the complexes ($p > 0.050$) and no significant G × T for complex IV ($p = 0.953$). In addition, there was no significant main effect of group ($p = 0.097$) or G × T ($p = 0.427$) for complex V. Protein levels of mitochondrial complexes in the pectoralis muscle can be found in figure 1*a*. Complex III was not detected with our antibody cocktail. Lastly, there were no significant main effects of group, time or G × T for the quadriceps muscle ($p > 0.050$ for all comparisons; exact *p*-values in figure 1*b*). Representative Western blots for mitochondrial complexes in the pectoralis and quadriceps can be found in figure 1*c*,*d*, respectively.

(c) Mitochondrial biogenesis

In the pectoralis muscle, migratory white-crowned sparrows had greater PGC-1α (0.33, ±95% CI [0.21], $p = 0.003$) and NRF1 (0.21, ±95% CI [0.13], $p = 0.003$) but lower TFAM (0.41, ±95% CI [0.19], $p < 0.001$) protein content compared with non-migratory white-crowned sparrows. There was no significant main effect of time ($p = 0.386$) or G × T ($p = 0.056$) for PGC-1α. There was a significant main effect of time for NRF1, in which NRF1 protein content was lower at baseline compared with pre-migration (0.25, ±95% CI [0.21], $p = 0.023$) and mid-migration (0.34, ±95% CI [0.18], $p > 0.001$). A significant G × T interaction was also found for NRF1, in which the migratory white-crowned sparrows had greater NRF1 protein content at Pre- (0.44, ±95% CI [0.31], $p = 0.001$) and mid-migration (0.69, ±95% CI [0.31], $p < 0.001$) compared with baseline. NRF1 protein content was also greater in migratory white-crowned sparrows at mid-migration compared with non-migratory white-crowned sparrows at baseline (0.52, ±95% CI [0.31], $p = 0.023$) and at mid-migration (0.53, ±95% CI [0.31], $p < 0.001$). In addition, there was no significant main effect of time ($p = 0.983$) or G × T ($p = 0.803$) for TFAM. Protein levels of mitochondrial biogenesis markers in the pectoralis muscle can be found in figure 2*a*. Lastly, there were no significant main effects of group, time or G × T for the quadriceps muscle ($p > 0.050$ for all comparisons; exact *p*-values can be found in figure 2*b*). Representative Western blots for mitochondrial biogenesis markers in the pectoralis and in the quadriceps can be found in figure 2*e*,*f*, respectively.

(d) Mitophagy

In the pectoralis muscle, migratory white-crowned sparrows had lower PINK1 protein content compared with non-migratory white-crowned sparrows (0.04, ±95% CI [0.03], $p = 0.009$). However, there was no main effect of time ($p = 0.953$) or G × T ($p =$

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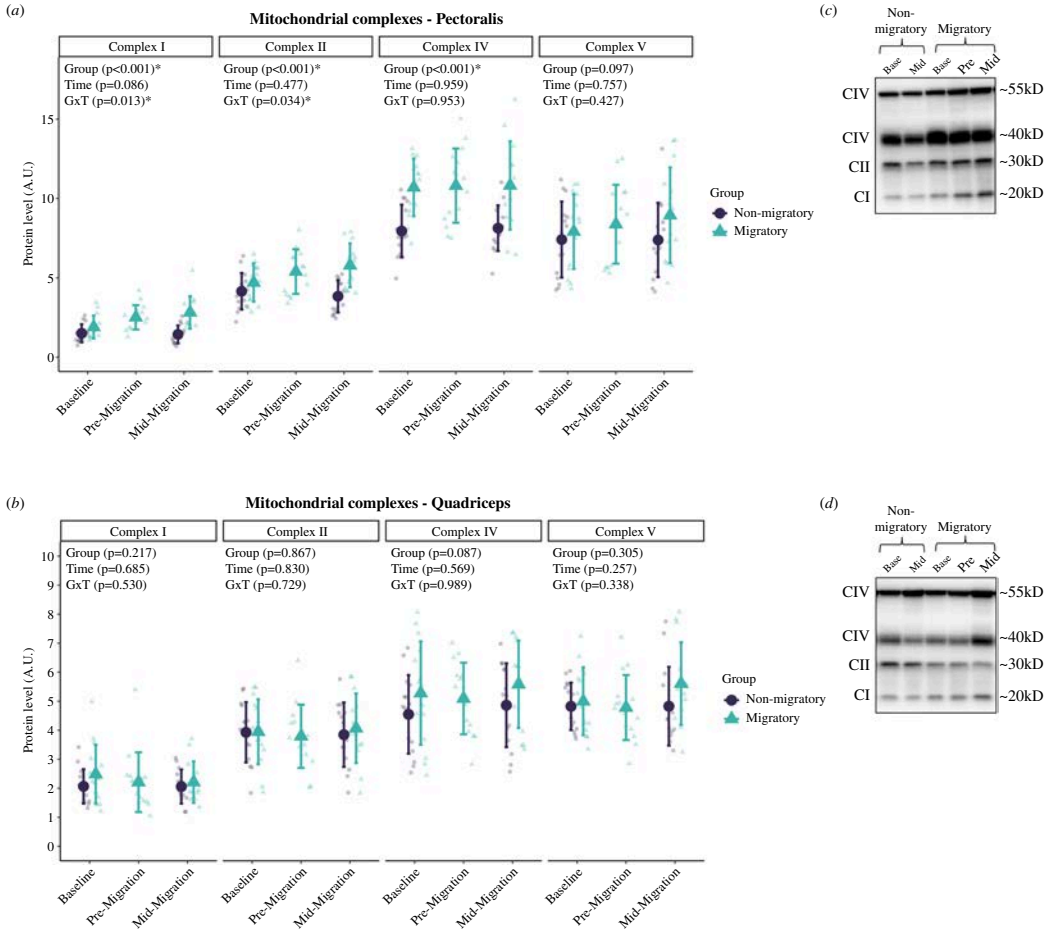

Figure 1. Protein levels of mitochondrial complexes I, II, IV and V in the pectoralis muscle (*a*) and quadriceps muscle (*b*) of non-migratory and migratory white-crowned sparrows at baseline (Base), pre-migration (Pre) and mid-migration (Mid). *statistically significant ($p < 0.050$). Representative Western blots for pectoralis and quadriceps, respectively (*c, d*). Sample size for pectoralis: $n = 15$ non-migratory white-crowned sparrows at baseline and mid-migration, $n = 15$ migratory white-crowned sparrows at baseline and pre-migration and $n = 14$ at mid-migration. Sample size for quadriceps: $n = 15$ non-migratory birds at baseline, $n = 14$ at mid-migration, $n = 15$ migratory white-crowned sparrows at baseline and pre-migration and $n = 14$ at mid-migration.

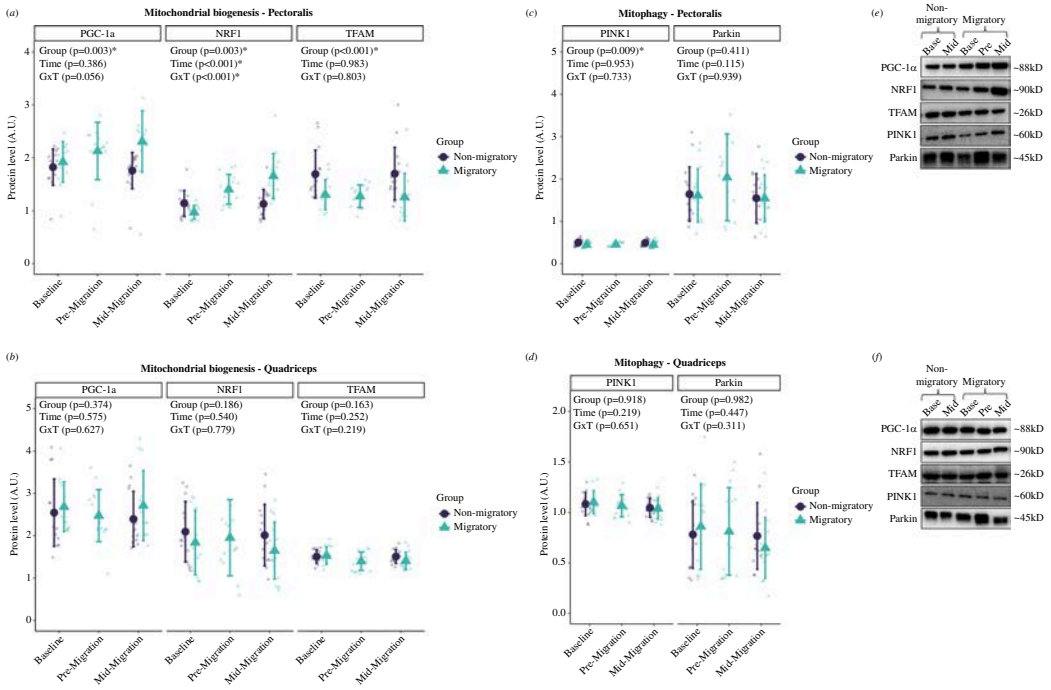

Figure 2. Protein levels of mitochondrial biogenesis and mitophagy in the pectoralis muscle (*a,c*) and quadriceps muscle (*b,d*) of non-migratory and migratory white-crowned sparrows at baseline (Base), pre-migration (Pre) and mid-migration (Mid). *statistically significant ($p < 0.050$). Representative Western blots for pectoralis and quadriceps, respectively (*e,f*). Sample size for pectoralis: $n = 15$ for both non-migratory and migratory white-crowned sparrows at all timepoints and every protein. Sample size for quadriceps: $n = 15$ non-migratory white-crowned sparrows at baseline, $n = 14$ at mid-migration, $n = 14$ migratory white-crowned sparrows at baseline and $n = 15$ at pre- and mid-migration.

0.733) for PINK1. In addition, there were no significant main effects of group ($p = 0.411$), time ($p = 0.115$) or G x T ($p = 0.939$) for Parkin. Protein levels of mitophagy markers in the pectoralis muscle can be found in figure 2*c*. In the quadriceps muscle, there were no significant main effects of group, time or G x T for either PINK1 or Parkin protein content ($p > 0.050$ for all comparisons; exact p-values can be found in figure 2*d*). Representative Western blots for mitophagy markers in the pectoralis and in the quadriceps can be found in figure 2*e,f*, respectively.

(e) Mitochondrial fusion

In the pectoralis muscle, there were no significant main effects of time ($p = 0.140$), group ($p = 0.481$) or G × T ($p = 0.117$) for Mfn1 protein content. Mfn2 protein content was greater in the pectoralis of migratory white-crowned sparrows compared with non-migratory white-crowned sparrows (group effect, 0.53, ±95% CI [0.44], $p = 0.020$). There was also a significant G × T for Mfn2 protein content, such that Mfn2 protein content was greater in migratory white-crowned sparrows at mid-migration compared with baseline (1.04, ±95% CI [1.00], $p = 0.037$) and compared with non-migratory white-crowned sparrows at mid-migration (1.03, ±95% CI [1.00], $p = 0.039$). There were significant main effects of group, time and G × T for OPA1. OPA1 protein content was greater in the pectoralis of migratory white-crowned sparrows compared with non-migratory birds (1.82, ±95% CI [0.31], $p < 0.001$). OPA1 protein content was greater at pre-migration (0.95, ±95% CI [0.66], $p = 0.003$) and at mid-migration (1.46, ±95% CI [0.54], $p < 0.001$) compared with baseline. In addition, OPA1 protein content was greater in the pectoralis of migratory white-crowned sparrows at pre-migration compared with baseline (1.61, ±95% CI [0.93], $p < 0.001$) and compared with non-migratory white-crowned sparrows at both baseline (2.11, ±95% CI [0.93], $p < 0.001$) and mid-migration (1.88, ±95% CI [0.93], $p < 0.001$). Further, OPA1 protein content was greater in the pectoralis of migratory white-crowned sparrows at mid-migration compared with both pre-migration (1.08, ±95% CI [0.93], $p = 0.014$) and baseline (2.68, ±95% CI [0.93], $p < 0.001$), and compared with non-migratory white-crowned sparrows at mid-migration (2.96, ±95% CI [0.93], $p < 0.001$) and baseline (3.18, ±95% CI [0.93], $p < 0.001$). Protein levels of mitochondrial fusion markers in the pectoralis muscle can be found in figure 3*a*. Lastly, there were no significant main effects of group, time or G × T for any of the markers investigated in the quadriceps muscle ($p > 0.050$ for all comparisons; exact p-values can be found in figure 3*b*). Representative Western blots for mitochondrial fusion markers in the pectoralis and in the quadriceps can be found in figure 3*e,f*, respectively.

(f) Mitochondrial fission

In the pectoralis muscle, there were significant main effects of group, time and G × T for Fis1. Fis1 protein content was greater in the pectoralis of migratory compared with non-migratory white-crowned sparrows (0.16,95% CI [0.08], $p < 0.001$). The protein content of Fis1 was greater at mid-migration compared with baseline (0.15,95% CI [0.11], $p = 0.006$). In addition, Fis1 protein content was greater in the pectoralis of migratory white-crowned sparrows at mid-migration compared with baseline (0.29,95% CI [0.19], $p < 0.001$), and compared with non-migratory white-crowned sparrows at both baseline (0.32,95% CI [0.19], $p < 0.001$) and mid-migration (0.32,95% CI [0.19], $p < 0.001$). There were also significant main effects of group, time and G × T for Drp1 in the pectoralis muscle. Drp1 protein content was greater in the pectoralis of migratory white-crowned sparrows compared with non-migratory hite-crowned Sparrows (0.59,95% CI [0.25], $p < 0.001$). The protein content of Drp1 was greater at mid-migration compared with baseline (0.51,95% CI [0.33], $p = 0.001$). In addition, Drp1 protein content was greater in the pectoralis of migratory white-crowned sparrows pre-migration compared with non-migratory white-crowned sparrows at both baseline (0.74,95% CI [0.57], $p = 0.004$) and mid-migration (0.59,95% CI [0.57], $p = 0.035$). Further, Drp1 protein content was greater in the pectoralis of migratory white-crowned sparrows at mid-migration compared with baseline (0.86,95% CI [0.57], $p < 0.001$), and compared with non-migratory white-crowned sparrows at both baseline (1.06,95% CI [0.57], $p < 0.001$) and at mid-migration (0.91,95% CI [0.57], $p < 0.001$). In the quadriceps muscle, there was a significant main effect of time for Fis1, such that Fis1 protein levels were greater at baseline compared with pre-migration (0.19,95% CI [0.19], $p = 0.049$). There were no significant main effects of group ($p = 0.489$) or G × T ($p = 0.668$). Protein levels of mitochondrial fission markers in the pectoralis muscle can be found in. Lastly, there were no significant main effects of group ($p = 0.555$), time ($p = 0.448$) or G × T ($p = 0.846$) for Drp1 in the quadriceps muscle. Representative Western blots for mitochondrial fission markers in the pectoralis and in the quadriceps can be found in figure 3*c,e,f*, respectively.

(g) Association between mitochondrial remodelling and mitochondrial respiratory function

Significant positive associations were found between pyruvate (P) + malate (M) + glutamate (G)-driven maximal respiration in the migratory white-crowned sparrows (from results reported in Rhodes *et al*. [6]) and pectoralis muscle protein content of complex I ($R^2 = 0.14$, $p = 0.011$, electronic supplementary material, figure S1-A), PGC-1α ($R^2 = 0.11$, $p = 0.021$, electronic supplementary material, figure S1-B), NRF1 ($R^2 = 0.29$, $p < 0.001$, figure 4*a*), OPA1 ($R^2 = 0.23$, $p = 0.001$, figure 4*b*), Fis1 ($R^2 = 0.26$, $p < 0.001$, figure 4*c*) and Drp1 ($R^2 = 0.10$, $p = 0.027$, electronic supplementary material, figure S1-C). Additionally, significant associations were found between succinate-driven maximal respiration and pectoralis muscle protein content of PGC-1α ($R^2 = 0.09$, $p = 0.038$, electronic supplementary material, figure S1-D), NRF1 ($R^2 = 0.12$, $p = 0.020$, electronic supplementary material, figure S1-F), OPA1 ($R^2 = 0.09$, $p = 0.042$, electronic supplementary material, figure S1-E) and Fis1 ($R^2 = 0.09$, $p = 0.042$, electronic supplementary material, figure S1-G). Lastly, significant associations were found between palmitoylcarnitine-driven maximal respiration and pectoralis muscle protein content of complex I ($R^2 = 0.18$, $p = 0.005$, electronic supplementary material, figure S1-H), PGC-1α ($R^2 = 0.15$, $p = 0.008$, electronic supplementary material, figure S1-J), NRF1 ($R^2 = 0.15$, $p = 0.008$, electronic

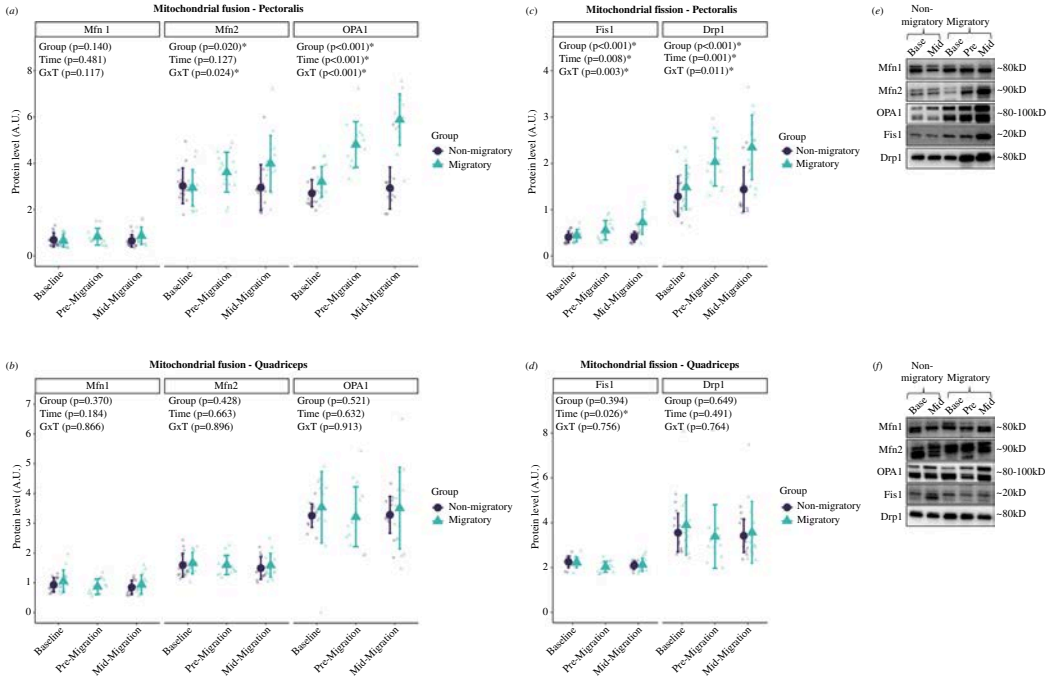

Figure 3. Protein levels of mitochondrial fusion and fission in the pectoralis muscle (*a*, *c*) and quadriceps muscle (*b*, *d*) of non-migratory and migratory white-crowned sparrows at baseline (Base), pre-migration (Pre) and mid-migration (Mid). *statistically significant ($p < 0.050$). Representative Western blots for pectoralis and quadriceps, respectively (*e*,*f*). Sample size for pectoralis: $n = 15$ for both non-migratory and migratory white-crowned sparrows at all timepoints and every protein. Sample size for quadriceps: $n = 15$ non-migratory white-crowned sparrows at baseline, $n = 14$ at mid-migration, $n = 14$ migratory white-crowned sparrows at baseline and $n = 15$ at pre- and mid-migration (exception for Drp1, for which sample size was $n = 15$ for non-migratory white-crowned sparrows at baseline and for migratory white-crowned sparrows at baseline and mid-migration, and $n = 14$ for non-migratory and migratory white-crowned sparrows mid-migratory.

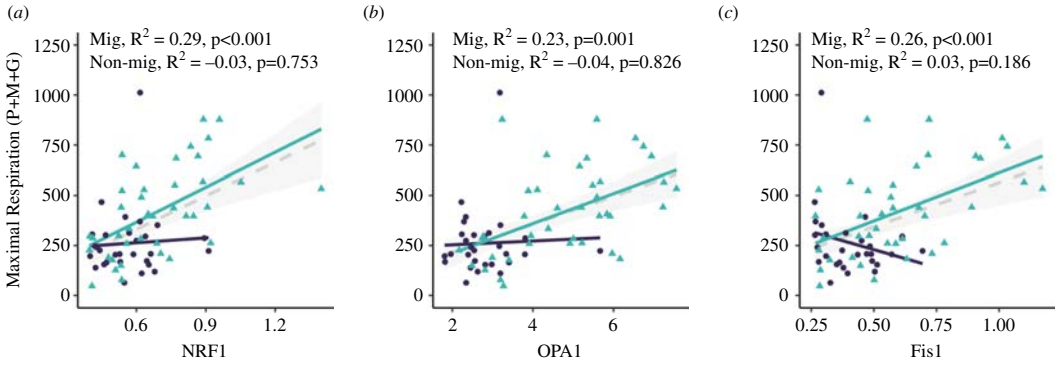

Figure 4. Significant relationships between pyruvate (P) + malate (M) + glutamate (G)-driven maximal respiration and pectoralis muscle protein contents of NRF1, OPA1 and Fis1 in migratory (Mig, green triangles) and non-migratory (Non-mig, dark blue circles) white-crowned sparrows. Individual values are depicted.

supplementary material, figure S1-L), OPA1 ($R^2 = 0.23$, $p = 0.001$, electronic supplementary material S1-I), Fis1 ($R^2 = 0.29$, $p < 0.001$, electronic supplementary material, figure S1-K) and Drp1 ($R^2 = 0.11$, $p = 0.023$, electronic supplementary material, figure S1-M). The only significant correlation between the non-migrant and maximal respiration was negative for Drp1 ($R^2 = 0.12$, $p = 0.038$). Significant associations can be seen in figure 4 and electronic supplementary material, figure S1.

4. Discussion

The goal of our study was to test the hypothesis that mitochondrial remodelling increases to meet the high energy demands of migration. Specifically, we were interested in testing whether patterns of mitochondrial remodelling would reflect fixed differences between migratory Gambel's white-crowned sparrow and non-migratory Nuttall's white-crowned sparrow or if differences would be a consequence of individual flexibility within migrants. Further, to determine whether changes are global (whole-body) or specific to muscles actively recruited during flight, we assessed both the pectoralis (active) and quadriceps ('inactive') muscles. Consistent with our hypothesis that mitochondrial remodelling plays a role in enabling high-energy activities such as migration, our results showed that individuals from the migratory population had greater overall mitochondrial remodelling compared with birds from the non-migratory population. Moreover, compared with non-migratory birds, individuals that migrated displayed flexibility, increasing several markers of mitochondrial remodelling between pre- and mid-migration periods. Importantly, although the highest levels of mitochondrial remodelling were observed during mid-migration, upregulation of mitochondrial remodelling started in anticipation of migration, aligning with the patterns

previously reported for mitochondrial respiratory function [6]. Further, the greater levels of mitochondrial remodelling and its upregulation during migration were specific to active muscles. Finally, non-migratory birds mostly had no changes in mitochondrial remodelling at timepoints corresponding to migration in the migratory species.w

A previous study found that migrants had higher citrate synthase activity in the pectoralis muscle compared with non-migrants, suggesting greater mitochondrial content in migrants [6]. In addition, migrants had greater mitochondrial content pre-migration compared with baseline. The protein contents of individual mitochondrial complexes have also been used as markers of mitochondrial content [23,41,42]. Therefore, the greater protein levels of complexes I, II and IV in the pectoralis muscle of migrants compared with non-migrants in the current study corroborate Rhodes *et al.*'s [6] findings. Our results also indicate that the upregulation of mitochondrial content is specific to active muscles because no differences were observed in the protein levels of mitochondrial complexes in the quadriceps muscle. Interestingly, we did not find significant differences in the levels of mitochondrial complex V. Given the greater mitochondrial respiratory function previously observed in migratory birds [6] and the fact that complex V is responsible for the synthesis of ATP, one could intuitively expect changes in levels of complex V. However, it is important to note that changes in protein levels alone do not necessarily lead to changes in enzymatic activity. Furthermore, a lack of change in skeletal muscle complex V protein content in response to exercise while other complexes increase has previously been observed in humans [24]. Nonetheless, the observed increase in mitochondrial content in the pectoralis muscle likely allows higher ATP production during migration while avoiding the unnecessary energetic cost of maintaining high mitochondrial content in tissues with limited activity, such as the quadriceps.

Mitochondrial content in skeletal muscle is determined by the balance between the production of new mitochondrial proteins (mitochondrial biogenesis) and degradation of existing, usually malfunctioning, mitochondria (mitophagy). Mitochondrial biogenesis is a complex process that involves both nuclear and mitochondrial genomes. PGC-1α is considered a master regulator of mitochondrial biogenesis. PGC-1α activates NRF1 and TFAM, increasing the transcription of mitochondrial genes encoded by the nuclear genome and driving transcription and replication of mitochondrial DNA, respectively [21,43,44]. Considering the greater mitochondrial content that we observed in the pectoralis of migrants, increased levels of mitochondrial biogenesis were expected. Indeed, we observed higher levels of PGC-1α and NRF1 in the pectoralis of migrants. In addition, migrants seemed to further upregulate mitochondrial biogenesis pre- and mid-migration. However, TFAM protein levels were surprisingly lower in the pectoralis of migrants compared with non-migrants. We can only speculate on why the patterns that we observed were the opposite of what we predicted. Our data suggest a preferential activation of nuclear genome to upregulate mitochondrial biogenesis in the active muscles of migratory birds. Future studies should investigate the differences in nuclear–mitochondrial interactions between migratory and non-migratory species to further elucidate this matter.

As previously mentioned, besides biogenesis, the control of mitochondrial content is also determined by mitophagy. Mitophagy is an essential process that not only determines mitochondrial content but also maintais mitochondrial quality, as it degrades damaged or dysfunctional regions of mitochondria [45]. Mitophagy is activated by the PINK1/Parkin pathway, in which PINK1 accumulates in the outer membrane of damaged mitochondria and recruits Parkin, which executes their degradation [46]. Mitophagy has been shown to increase in response to conditions of increased energy demand, such as exercise [45,47,48]. In addition, mitophagy can be triggered in response to oxidative stress [49] and different studies have reported increased oxidative stress in tissues of migrants [50–52]. Although it is likely that the majority of reactive oxygen species (ROS) produced during migration are of non-mitochondrial origin, non-mitochondrial ROS can still lead to mitochondrial damage [53,54]. Therefore, we expected greater mitophagy levels in the pectoralis of migrants, with further increases during migration. However, our results showed lower PINK1 protein levels in the pectoralis muscle of migrants compared with non-migrants, and no differences between timepoints. It is possible that the upregulation of the antioxidant defense system observed in migratory birds [55–57] prevents substantial mitochondrial damage and/or that migrants downregulate mitophagy to maintain higher mitochondrial content, although alternative mechanisms may also cause mitophagy to decline or remain unchanged. Lastly, it is important to note that although we found decreased PINK1 protein levels in migrants, mitophagy could have been upregulated through phosphorylation of PINK1 and Parkin [58] or through PINK1/Parkin-independent pathways.

Mitochondrial morphology can be regulated by fusion and fission events, usually termed mitochondrial dynamics. The relationship between form and function is observed across different fields of biology and mitochondrial dynamics act not only to regulate mitochondria morphology, but are also important determinants of mitochondrial function and ultimately, cell and organism health [22,59,60]. Mitochondrial fusion can promote the exchange and complementation of different components between mitochondria and involves Mfn1 and Mfn2, which control the fusion of the outer mitochondrial membrane, and Opa1, which mediates the fusion of the inner mitochondrial membrane (IMM; [59]). On the other hand, fission segregates damaged portions of mitochondria that can be further eliminated through mitophagy and is carried out by Drp1, which is recruited from the cytosol by Fis1. Our results showed greater levels of both mitochondrial fusion and fission in the pectoralis muscle of migrants, with further increases before and during active migration. Mitochondrial morphology is highly variable in different tissues and in its response to different stressors [20,61]. The concomitant increase of both mitochondrial fusion and fission has also been reported in the skeletal muscle of humans after exercise [23,62]. Collectively, our results show an increase in overall mitochondrial dynamics in the pectoralis muscle, but without imaging techniques, we are unable to determine whether the final outcome was a more connected or fragmented mitochondrial network.

Identifying the precise relationship between mitochondrial morphology and oxidative phosphorylation has been challenging owing to the complex relationship between the dynamics of mitochondria and other organelles, such as the endoplasmic reticulum and peroxisome [63]. Given that fission and fusion, in particular, are known to play important roles in maintaining mitochondrial respiratory function [17,18,63] and that several markers increase with an improved capacity for oxidative phosphorylation with regular exercise [23,25,29], we propose that mitochondrial remodelling modulates skeletal muscle oxidative phosphorylation in wild birds [15]. When we evaluated the relationship between mitochondrial dynamics

markers and respiration measured by Rhodes *et al*. [6], we found that several mitochondrial dynamics markers are significantly correlated with maximal respiration in migrants. In particular, when provided with either a cocktail of pyruvate, malate and glutamate substrates or a palmitoylcarnitine substrate—both of which generate $NADH^{+}$ for complex I respiration—both fusion marker OPA1 and fission marker Fis1 explained more than 20% of the variation in maximal respiration among migrant individuals. These markers also displayed a positive relationship with maximal respiration with succinate as substrate, which generates $FADH^{2+}$. PGC-1α and NRF1, which both play a role in upregulating mitochondrial biogenesis, also increase with increased maximal respiration. Similar patterns were not observed in the non-migrants. It is worth noting that these patterns occurred in spite of the fact that the mitochondria were isolated from the tissue, removing them from the native configuration of the mitochondria in the cells [6]. These results highlight the importance of mitochondrial dynamics to mitochondrial respiratory function in Gambel's white-crowned sparrow migrants and suggest that mitochondrial dynamics could play a role in the changes in respiratory performance observed with this subspecies.

In summary, our study is the first to show that mitochondrial remodelling supports migration in Gambel's white-crowned sparrows. Gambel's white-crowned sparrows showed both fixed and flexible remodelling phenotypes, with greater overall mitochondrial remodelling compared with the non-migratory Nuttall's subspecies but further upregulating mitochondrial remodelling throughout migration. In addition, the observed differences in mitochondrial remodelling were specific to a muscle that is actively recruited during flight, highlighting a highly specific and efficient phenotype to support the increased energetic demands of migration. There are limitations to the current study. First, even though we detected differences in the protein levels of different markers of mitochondrial dynamics, as the name suggests, these are dynamic processes and measuring protein levels only provides a 'snapshot'. Further, the remodelling of the mitochondrial network involves other proteins and processes (e.g. post-translational modifications) that were not investigated herein. Future studies could benefit from different omics approaches to identify different levels of control of mitochondrial remodelling during migration and from imaging techniques to identify the state of the mitochondrial network and changes in different mitochondrial structures, such as IMM density and intermitochondrial junctions.

Ethics. The current study was approved by the Institutional Animal Care and Use Committee (IACUC) at Auburn University (PRN #2019-3549). Birds were collected according to Migratory Bird Permit #MB49547D and California state permit #210190009-21019-001.

Data accessibility. The data and codes supporting this paper are freely available at Dryad [64].

Supplementary material is available online [65].

Declaration of AI use. We have not used AI-assisted technologies in creating this article.

Authors' contributions. P.H.C.M.: conceptualization, data curation, formal analysis, investigation, methodology, writing—original draft, writing—review and editing; E.M.R.: conceptualization, data curation, formal analysis, investigation, methodology, writing—review and editing; K.N.Y.: conceptualization, data curation, investigation, methodology, writing—review and editing; B.J.M.: data curation, formal analysis, investigation, methodology, writing—review and editing; G.E.H.: conceptualization, data curation, funding acquisition, investigation, methodology, project administration, writing—review and editing; W.R.H.: conceptualization, data curation, funding acquisition, investigation, methodology, project administration, writing—review and editing; A.N.K.: conceptualization, data curation, funding acquisition, investigation, methodology, project administration, writing—review and editing.

All authors gave final approval for publication and agreed to be held accountable for the work performed therein.

Conflict of interest declaration. We declare we have no competing interests.

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