



Seasonal modulation of pectoralis muscle fiber type composition in migratory songbirds

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Received: 2 October 2023 / Revised: 22 February 2024 / Accepted: 17 April 2024
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Abstract

The pectoralis major is the muscle required for migratory flight in songbirds, and has been believed to be exclusively composed of fast oxidative glycolytic (FOG) fibers in most small songbirds (< 20 g). Here, we investigated the effect of season (migratory versus non-migratory) and migratory distance (within North America versus to South America) on muscle fiber type in three songbird families: vireos (Vireonidae), warblers (Parulidae), and thrushes (Turdidae). FOG and fast glycolytic (FG) fibers were identified using myosin-ATPase staining. Short-distance migrants within the vireo and warbler families altered their pectoralis muscle to contain FG fibers during non-migratory conditions, while long-distance migrants maintained exclusively FOG fibers, regardless of season. Thrushes, a family of larger songbirds, exhibited mixed fibers regardless of season or migratory distance. This study is one of the first to identify FG fibers in small North American songbirds and highlights the potential role of migratory distance and season on muscle phenotype.

Keywords Myosin ATPase staining · Fiber density · Migration distance · Seasonal plasticity

Zusammenfassung

Jahreszeitlich bedingte veränderte Zusammensetzung der Brustmuskelfasern bei ziehenden Singvögeln

Der Brustmuskel (*Pectoralis major*) ist für den Flug ziehender Singvögel ausschlaggebend, und man geht bislang davon aus, dass er bei den meisten kleinen Singvögeln (< 20 g) ausschließlich aus schnellen oxidativ-glykolytischen Fasern (FOG) besteht. Wir untersuchten den Einfluss der Jahreszeit (Zugzeit versus Nicht-Zugzeit) und der Zugdistanz (innerhalb Nordamerikas gegenüber nach Südamerika) auf den Muskelfasertyp bei drei Singvogelfamilien: Vireos (Vireonidae), Waldsänger (Parulidae), und Drosseln (Turdidae). FOG und schnelle glykolytische (FG) Fasern wurden durch das Anfärben der Myosin-ATPase identifiziert. Bei den Kurzstreckenziehern aus den Familien der Vireo und der Waldsänger veränderte sich der Brustmuskel dergestalt, dass er zwischen den Zugzeiten FG-Fasern enthielt, während die Langstreckenzieher unabhängig von der Jahreszeit ausschließlich die FOG-Fasern beibehielten. Die Drosseln als Familie größerer Singvögel wiesen unabhängig von der Jahreszeit oder der Zugentfernung gemischte Fasertypen auf. Diese Studie ist eine der ersten, in der FG-Fasern bei kleinen nordamerikanischen Singvögeln nachgewiesen wurden, und sie unterstreicht die mögliche Rolle der Zugdistanz und der Jahreszeit für den Muskelphänotyp.

Introduction

Migratory flight is regarded to be one of the most physiologically demanding migratory strategies, in terms of energy expenditure and physical stress. Birds that migrate require high endurance to maintain flight for multiple hours and up to many consecutive days, which can be more than twice as aerobically demanding as running (Butler 1991). Migratory bird species have been reported to increase their endurance and aerobic capacity by adjusting their physiology and biochemistry during pre-migration and migration to

Communicated by I. Moore.

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support their journey. These changes can involve increases in fat utilization, improvements to cardiovascular function, and increases in pectoralis mass (Blem 1976; Marsh 1984; Driedzic et al. 1993; Guglielmo 2010), but whether there are also associated changes in flight muscle fiber type is unknown.

The pectoralis major is the dominant muscle responsible for flight and makes up the majority of the muscle mass in birds (Jimenez 2020). It assists with generating lift and thrust while supporting the functional demands of sustained flight (Driedzic et al. 1993). There are three main types of twitch fibers within the avian musculature. Slow oxidative (SO) fibers contract muscles at slow velocities without fatigue, but produce less force than other fiber types (Welch and Altshuler 2009). Flightless, soaring, and gliding birds commonly have SO (Rosser et al. 1994; Meyers 1997), which aid in maintaining posture and sustained muscle contraction (Goldspink 1980). Fast glycolytic (FG) fibers are anaerobic and contain few mitochondria (Peter et al. 1972). They exhibit high contraction dynamics to generate high force for short durations; however, they lack endurance (Welch and Altshuler 2009). Thus, these fibers are more suitable for brief bursts of high-powered flight, and are more commonly observed in volant and larger birds (> 30 g) (Rosser and George 1986; Lundgren and Kiessling 1988). In contrast, fast-oxidative glycolytic (FOG) fibers are fatigue-resistant fibers that utilize aerobic metabolism and can maintain high contraction frequency for long durations (Peter et al. 1972). FOG fibers are assumed to comprise most of the flight muscle fibers in smaller birds (< 20 g), as this aids in high flap-powered flight and can sufficiently fulfil many aspects of bird flight (e.g., perching, burst responses, prolonged flight) (Torrella et al. 1998; Welch and Altshuler 2009). In contrast to this assumption, Ovenbirds (*Seiurus aurocapilla*) (Rosser and George 1986), European Robins (*Erithacus rubecula*) (Lundgren and Kiessling 1988), Bush Robins (*Tarsiger rufilatus*, *Tarsiger indicus*, *Tarsiger chrysaeus*) (DuBay et al. 2020), and the Eurasian Tree Sparrow (*Passer monanus*) (Qu et al. 2020), all which are < 20 g in mass, have FOG and FG fibers present in their pectoralis muscle. These findings indicate that some smaller birds may have FOG and FG fibers present in their pectoralis muscles.

The flight muscle has been observed to exhibit plasticity between migratory and non-migratory periods, but the magnitude of this plasticity may be dependent on migratory distance. Increases in flight muscle mass and muscle fiber diameter have been reported in various migratory songbirds and shorebirds leading up to migration (Marsh and Storer 1981; Marsh 1984; Butler and Turner 1988; Lindström et al. 2000; Bauchinger and Biebach 2005; Vézina et al. 2021), which would be important for increasing the maximal power of the flight muscle for migration (Driedzic et al. 1993). Lipid-oxidative capacity of the flight muscle is also greater

during migration compared to non-migratory periods (Saunders and Klemm 1994; Guglielmo et al. 2002; Dick 2017; Guglielmo 2018), suggesting that there could be changes in fiber-type composition of the flight muscle in some migratory birds. For birds that migrate long distances across major ecological barriers, such as deserts or oceans, the pectoralis muscle has been observed to be primarily composed of FOG fibers, to have greater capillary densities, and to be catabolized more during migratory flight compared to species that conduct nocturnal migration with many stop-over sites (Rosser and George 1986; Lundgren and Kiessling 1988; Bauchinger and Biebach 2005).

The objective of this study was to investigate whether FG and FOG fiber types were present in six songbird species from three different families. We hypothesized that fiber type composition of the flight muscle would be influenced by (1) season (migratory versus non-migratory), and (2) migratory distance (within North America versus between Canada and South America). We used migratory songbirds (Order Passeriformes) to investigate these hypotheses as Passeriformes exhibit variation in migratory distance within families, but also variation in body size across families. Species from Vireonidae (vireos), Parulidae (warblers), and Turdidae (thrushes) were used for this study as they are known to commonly migrate through Southern Ontario, Canada and have varying migratory distances within families. Warbling Vireos (*Vireo gilvus*), Myrtle Yellow-rumped Warblers (*Setophaga coronata*), and Hermit Thrushes (*Catharus guttatus*) all migrate within North America, while Red-eyed Vireos (*Vireo olivaceus*), Blackpoll Warblers (*Setophaga striata*), and Swainson's Thrushes (*Catharus ustulatus*) migrate to South America. Our previous research on these species has shown that there is seasonal plasticity in muscle fiber transverse area in the short-distance migrants, with fall migrating birds having smaller transverse areas compared to non-migratory birds (Ivy and Guglielmo 2023). Whether these changes in fiber transverse area are due to changes in muscle fiber type is not known.

Methods

Songbirds and experimental design

All juvenile songbirds were captured during their south-bound migration at Long Point, Ontario, Canada, a stop-over site. Myrtle Yellow-rumped Warblers (N = 12) were caught in September 2020, while Warbling Vireos (N = 12), Red-eyed Vireos (N = 11), Blackpoll Warblers (N = 15), Hermit Thrush (N = 16), and Swainson's Thrush (N = 16) were caught in August and September 2021. All birds were housed at the Advanced Facility for Avian Research (London, Ontario, Canada) in free-flight aviaries, and fed

a house-made agar-based diet (Dick and Guglielmo 2019) supplemented with mealworms (*Tenebrio molitor*) and had unlimited access to water. Initially birds were kept on a natural fall photoperiod (12.5 h light: 11.5 h dark), with half of the birds of each species sampled (referred to as migratory). The remaining birds were transitioned to a short-day photoperiod (9 h light: 15 h dark) by mid-November and after 90 days in this photoperiod were sampled (referred to as non-migratory). These birds were part of a previous study looking at seasonal changes in ventilatory, haematological, and muscle histology parameters (Ivy and Guglielmo 2023). Animal capture and study procedures were approved by the University of Western Ontario Animal Care Committee (Protocol 2018-092) and the Canadian Wildlife Service (SC-OR-2018-0256).

Pectoralis fiber typing

Muscle fiber type was examined in the pectoralis muscle of all birds using immunohistochemistry techniques that have previously been described (Scott et al. 2009; DuBay et al. 2020). Pectoralis muscle samples ($\sim 0.5\text{--}1\text{ cm}^3$) had previously been taken from the middle of the muscle and spanned from the subcutaneous surface to the sternum (Ivy and Guglielmo 2023). These samples only contained the pectoralis muscle and total pectoralis muscle mass was taken as the sum of the mass of both pectoralis sides. Samples were mounted on cork and coated in mounting medium (Cryomatrix; Thermo Fisher Scientific, Waltham, MA, USA), frozen in liquid N_2 -cooled isopentane, and sectioned at $12\text{ }\mu\text{m}$ transverse to the muscle fiber length in a cryostat at $-20\text{ }^\circ\text{C}$. Slides were then air-dried and stored at $-80\text{ }^\circ\text{C}$ until staining.

Muscle fiber types were determined by staining for myosin-ATPase activity to identify FOG and FG fibers, as has previously been used in geese and songbirds (Scott et al. 2009; DuBay et al. 2020). Briefly, sections were brought to room temperature, and preincubated in an acidic incubation solution (100 mM sodium acetate, 10 mM EDTA, pH 4.3) for 3 min. After rinsing in dH_2O , slides were incubated in ATPase incubation buffer (200 mM tris, 18 mM CaCl_2 , 2.7 mM ATP, pH 9.5) for 20 min with gentle agitation. Slides were then rinsed for 15 min in CaCl_2 washing solution (1% w/v) and incubated in CoCl_2 solution (2% w/v) for 10 min with gentle agitation. Following rinsing in dH_2O , slides were developed in ammonium sulfide solution (2% w/v) for 30 s, rinsed in dH_2O , and mounted with Aquamount (Thermo Fisher Scientific, Waltham, MA, USA).

Muscle fiber types were confirmed by staining for succinate dehydrogenase (SDH) activity, as previously described (Scott et al. 2009; DuBay et al. 2020). Briefly, slides were incubated in a working buffer (0.6 mM nitroblue tetrazolium, 2.0 mM KH_2PO_4 , 15.4 mM Na_2HPO_4 , 16.7 mM sodium

succinate) for 60 min at $41\text{ }^\circ\text{C}$ in the dark. Slides were then rinsed in distilled water for 3 min and post-fixed in a sucrose-formol solution (159 mM sucrose, 3.7% formaldehyde) for 2 min. Slides were then washed thoroughly in distilled water and cover slipped with Aquamount (ThermoFisher Scientific).

Sections were imaged using light microscopy on a Leica microscope (CTR6500) with Leica Application Suite imaging software. For myosin-ATPase stained slides, stereological methods were used to make unbiased measurements (Egginton 1990; Lui et al. 2015). Images were collected such that there was an equal representation across the entire muscle cross-section. Preliminary analyses indicated that 12–16 images for each section sufficiently accounted for fiber-type heterogeneity established by the number of images required to produce a stable mean value for each individual. All images were manually analyzed and counted for each fiber type in ImageJ software (version 1.53). Fiber type counts were not conducted on the slides stained for SDH activity, as they were used to confirm the presence of FOG and FG staining in each species.

Statistical analyses

Fiber type densities were analyzed using two-way ANOVAs to examine the main effects of season (migratory vs. non-migratory) and migratory distance (within North America vs. to South America) within each family (Fig. 1). Holm-Sidak post-tests were conducted as appropriate. All statistical analysis was conducted with R, version 4.2.0 (R Foundation for Statistical Computing, Vienna, Austria). All values are repeated as mean \pm SEM and a significance level of $P < 0.05$ was considered statistically significant.

Results

Season and migratory distance significantly influenced fiber type densities in our songbirds (Figs. 2, 3). In vireos, FOG density was not significantly altered with season ($F_{1,19} = 2.663$, $P = 0.119$) or migratory distance ($F_{1,19} = 0.451$, $P = 0.510$; Fig. 3A). FG fibers were only present in Warbling Vireos (main effect of distance: $F_{1,19} = 4.575$, $P = 0.046$), regardless of migratory condition ($F_{1,19} = 0.001$, $P = 0.994$; Figs. 2A, B, 3B), but not Red-eyed Vireos. Not all Warbling Vireo pectoralis muscles sampled contained FG fibers, with only 3/6 migratory and 2/6 non-migratory individuals containing FG fibers, and only $\sim 4\%$ of the total fibers counted being FG fibers.

Similarly, warblers did not exhibit any significant changes in FOG density with season ($F_{1,23} = 1.590$, $P = 0.220$) or migratory distance ($F_{1,23} = 0.968$, $P = 0.335$; Fig. 3A). FG fibers were only present in non-migratory Yellow-rumped

Fig. 1 Representative depiction of the phylogenetic relationship of species used in this study from BirdTree (Jetz et al. 2012). Scientific name and common name are included for each species, followed by whether the species migrates within North America (short distance, SD) or to South America (long distance, LD)

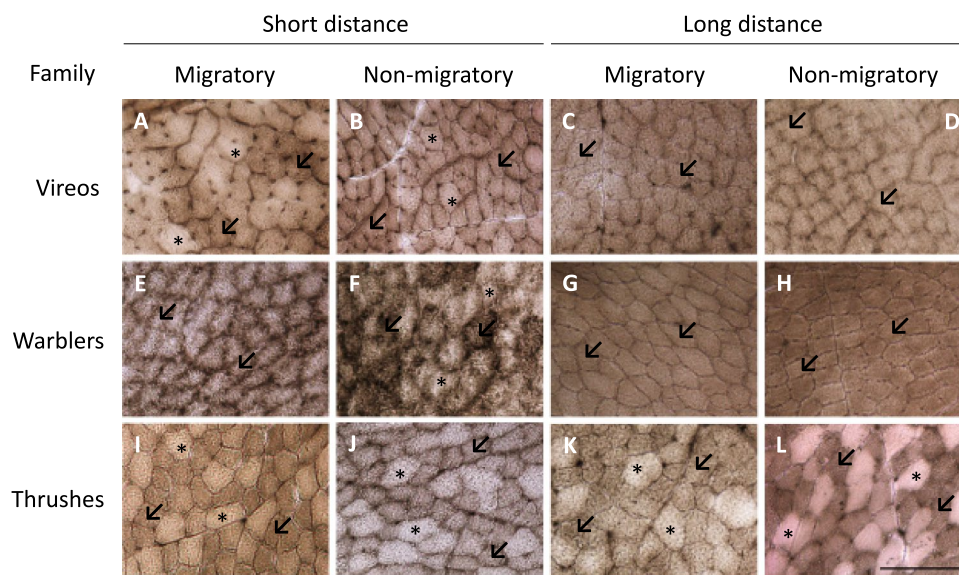
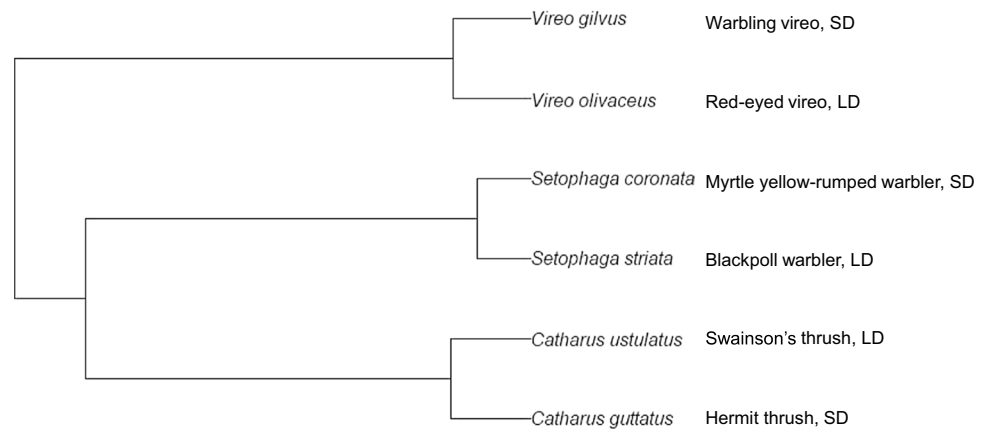


Fig. 2 Representative images of the pectoralis muscle of Warbling Vireos (A, B), Red-eyed Vireos (C, D), Myrtle Yellow-rumped Warblers (E, F), Blackpoll Warblers (G, H), Hermit Thrushes (I, J), and Swainson's Thrushes (K, L) during migratory (A, C, E, G, I, K) and non-migratory conditions (B, D, F, H, J, L). Short-distance species

are Warbling Vireos, Myrtle Yellow-rumped Warblers, and Hermit Thrushes; long-distance migrants are Red-eyed Vireos, Blackpoll Warblers, and Swainson's Thrushes. Myosin-ATPase staining was used to identify fast-oxidative glycolytic fibers (arrows) and fast-glycolytic fibers (asterisks). Scale bar = 100 μ m

Warblers (distance \times season interaction: $F_{1,23} = 6.414$, $P = 0.0186$, main effect of season: $F_{1,23} = 5.011$, $P = 0.035$, main effect of distance: $F_{1,23} = 8.886$, $P = 0.007$), but not at all in Blackpoll Warblers (Figs. 2E, F, 3B). Almost all non-migratory Yellow-rumped Warbler pectoralis muscles contained FG fibers (6/7 individuals), with $\sim 11\%$ of the total fibers counted being FG fibers.

In contrast, both hermit and Swainson's Thrush pectoralis muscles contained FOG and FG fibers (Figs. 2, 3A, B). A significant interaction between season and distance was observed with FOG fiber density ($F_{1,28} = 4.419$, $P = 0.045$), with migratory Swainson's Thrush having a 1.4-fold

higher FOG density compared to non-migratory Swainson's Thrush and hermit thrush in general (Fig. 3A). FG fiber density was also influenced by an interaction between season and distance ($F_{1,28} = 4.828$, $P = 0.036$), but post-hoc testing was unable to identify statistically different comparisons (Fig. 3B). Regardless, FG fibers were present in all migratory Hermit Thrush and 7/8 non-migratory Hermit Thrush, with $\sim 13\%$ and 8% of total fibers counted being FG fibers, respectively. In Swainson's Thrush, FG fibers were present in 6/8 migratory individuals and all wintering individuals, with $\sim 8\%$ and $\sim 17\%$ of total fibers being FG fibers, respectively.

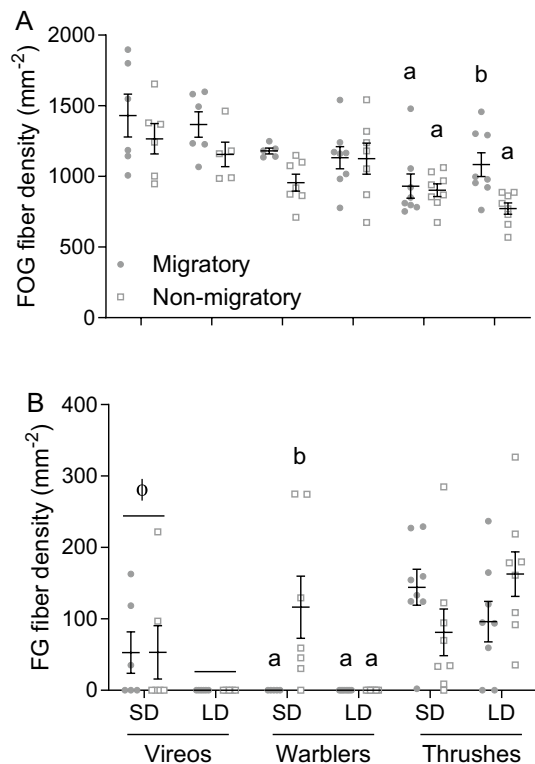


Fig. 3 Fast-oxidative glycolytic (FOG, **A**) and fast-glycolytic (FG, **B**) fiber densities in the pectoralis muscle of vireos, warblers, and thrushes during migratory (filled symbols) and non-migratory (open symbols) conditions. FG fiber densities were significantly influenced by migratory distance in vireos, and migratory distance and migratory condition in warblers. Short-distance migrants (SD) included Warbling Vireos, Myrtle Yellow-rumped Warblers, and Hermit Thrush; long distance migrants (LD) included Red-eyed Vireos, Blackpoll Warblers, and Swainson's Thrush. Individual values are plotted with mean \pm SEM, ϕ represents a significant main effect of migratory distance within a family, and groups within a family that do not share a letter are significantly different through pairwise comparisons after two-factor ANOVAs within each family. N=migratory, non-migratory, Warbling Vireo=6,6, Red-eyed Vireo=6,5, Myrtle Yellow-rumped Warbler=5,7, Blackpoll Warbler=8,7, Hermit Thrush=8,8, Swainson's Thrush=8,8

Discussion

Many past studies have assumed that the pectoralis muscle of small songbirds was composed exclusively of FOG fibers, as FOG fibers would support all flight demands (Rosser and George 1986; Welch and Altshuler 2009; Dakin et al. 2018). This claim was questioned in a recent study observing the presence of FOG and FG fiber types in the pectoralis muscles of bush robins (DuBay et al. 2020). Additionally, we have recently shown that fiber transverse areas in the flight muscle can change seasonally in songbird species that migrate within North America, suggesting that there could be changes in muscle fiber type composition (Ivy and Guglielmo 2023). Here, we show that Warbling Vireos, Myrtle

Yellow-rumped Warblers, Hermit Thrushes, and Swainson's Thrushes all have FG fibers. We also provide some of the first evidence that FG fiber density is influenced by season (migratory versus non-migratory) and potentially, migratory distance (within North America or to South America).

Changes in fiber type composition with migratory distance and season

FG fibers appeared to be influenced by migratory distance in vireos and warblers, but not thrushes. Warbling Vireos and Myrtle Yellow-rumped Warblers, which migrate within North America, were observed to have FG fibers present regardless of migratory period or only during non-migratory conditions, respectively, whereas both thrush species contained FG fibers. The changes in fiber type composition support the changes in fiber transverse area we previously observed in these species and the lack of change in fiber transverse area in Red-eyed Vireos and Blackpoll Warblers (Ivy and Guglielmo 2023). These differences in fiber type composition may be due to different energetic demands associated with short- versus long-distance migratory flight. For example, long-distance migrants not only fly for longer periods, but also make less frequent stops during migration compared to short-distance migrants. This strategy is costly and more energy intensive, therefore requiring more oxidative fibers to increase aerobic capacity (DuBay et al. 2020). Catabolism of the skeletal muscles for other needs other than for energy, such as obtaining water and maintaining blood glucose levels (Bauchinger and Biebach 2005) is also likely to occur. In these cases, it may be beneficial to maintain a uniform fiber type, so as not to compromise overall muscle function.

Alternatively, short-distance migrants make multiple stops during migration and are able to feed and drink more often, possibly allowing for flexibility in muscle fiber type composition. FG fibers are typically associated with take-off (Dial et al. 1987), so the incorporation of FG fibers in some of our migratory Warbling Vireos may suggest a different functional role. It is possible that these FG fibers may be present to aid in other forms of high-intensity flight, such as those associated with predator avoidance and/or competitive interactions, as was suggested for the presence of FG fibers in *Tarsiger* bush-robins (DuBay et al. 2020). Warbling Vireos breed in our region of southern Ontario. So, although these birds were caught at a common migratory stop-over site, the low proportion of Warbling Vireos with FG fibers during migratory conditions could be the result of changes in pectoralis morphology associated with breeding conditions. High-intensity flight would be important for predator avoidance and nest protection during the breeding season.

We observed changes in fiber type composition between migratory and non-migratory conditions. FG fibers were

identified in non-migratory Warbling Vireos, Myrtle Yellow-rumped Warblers, Hermit Thrushes, and Swainson's Thrushes, but not Red-eyed Vireos or Blackpoll Warblers. The appearance of FG fibers in non-migratory Myrtle Yellow-rumped Warblers is a novel finding and suggests that migratory phenotypes can play an important role in pectoralis morphology. The lack of FG fibers during migratory conditions highlights the importance of FOG fibers for migratory flight, as lipids would be the primary fuel source (Guglielmo 2018). The presence of FG fibers during non-migratory conditions could be the result of changes in foraging behaviour and predator avoidance strategies, therefore requiring the inclusion of FG fibers for burst movements (Dial et al. 1987). Although Warbling Vireos, Myrtle Yellow-rumped Warblers, and thrushes do not over-winter in particularly cold climates, having FG fibers during the non-migratory season could also be important for thermogenesis in the wintering range within North America (Swanson and Vézina 2015; Pani et al. 2023).

The pectoralis muscle of Blackpoll Warblers and Red-eyed Vireos only had FOG fibers, regardless of season. These findings suggest that incorporation of FG fibers may not be necessary during the non-migratory season for these species. Given that these birds conduct southbound migration to warm regions in South America, the need for FG fibers for thermogenesis would be minimal, suggesting that FOG fibers would be able to support the daily thermoregulatory and flight demands of these birds (Rosser and George 1986; Welch and Altshuler 2009; Dakin et al. 2018). We do acknowledge that our experimental design may confound species with migratory distance, as we are unable to disentangle migratory distance from random or neutrally evolving species differences. Further studies comparing more species that conduct short- and long-distance migration within a family would provide greater insight into our findings.

Hermit Thrushes and Swainson's Thrushes both had FOG and FG fibers in the pectoralis, regardless of season. Although we found a significant interaction between season and species in our study, a post hoc test was unable to identify statistically different comparisons. This suggests that there may still be some inconsistent changes that occur seasonally between the species, as Swainson's Thrushes appear to have a trend for decreases and increases in FOG and FG fiber density, respectively, in the non-migratory season, while Hermit Thrushes only exhibit a trend for decreasing FG density in the non-migratory season. This pattern is not what we would predict, given the pattern we saw in vireos and warblers, suggesting that pectoralis size may play a role in FG density (Rosser and George 1986). In our study, thrushes maintained a slightly higher proportion of FG fibers overall ~10% compared to vireos and warblers, which may suggest a threshold for the amount of FG fibers that are needed in these species of larger size. Whether larger

songbird species have a greater proportion of FG fibers or if ~10% is a limit for the proportion of FG fibers is unknown and requires further research.

In conclusion, we identified FG fibers in the pectoralis muscle of vireos and warblers that are less than 20 g. Our findings suggest that small songbirds that migrate to South America maintain a fiber type composition that is purely FOG, while those that migrate within North America can exhibit seasonal inclusion of FG fibers. We also observed thrushes to have FG and FOG fiber types present regardless of season or migratory distance, suggesting that FOG and FG fibers may be required to support flight demands of songbirds greater than 30 g. These findings highlight the importance of phenology and migratory distance on muscle physiology.

Acknowledgements The authors would like to thank staff at the Long Point Bird Observatory for their assistance with bird catching, Dr. Morag Dick, Soren Coulson, Kevin Young, and Corrine Genier for their help with animal transport and care, and Francis Boon and Michela Rebuli for support at the AFAR. We would also like to thank Dr. Scott MacDougall-Shackleton for the use of his cryostat and microscope. We acknowledge that Western University is located on the territories of the Anishinaabek, Haudenosaunee, Lūnaapéewak and Chonnonton Nations, on lands connected with the London Township and Sombra Treaties of 1796 and the Dish with One Spoon Covenant Wampum where this research was conducted and publication was written.

Author contributions C.M.I. and C.G.G. designed the study. R.M.C. carried out histological measurements and analyzed the data. R.M.C., C.M.I., and C.G.G. wrote and edited the manuscript.

Funding This research was funded by a Natural Sciences and Engineering Research Council of Canada (NSERC) Discovery Grant to C.G.G. (RGPIN-2020-07204). Salary support was provided to C.M.I. by a NSERC PDF and Banting Scholarship.

Data availability Data is deposited in Mendeley Data: doi: <https://doi.org/10.17632/r7mcpdmgbv.1>

Declarations

Competing interest The authors declare no competing interests.

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