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ORIGINAL ARTICLE

Seasonal and flight-related variation of galectin expression in heart, liver and flight muscles of yellow-rumped warblers (*Setophaga coronata*)

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Abstract Galectins, a family of multifunctional glycan-binding proteins, are proposed as biomarkers of cellular stress responses. Avian migration is an energetically challenging physical stress, which represents a physiological model of muscular endurance exercises. This study assesses change in galectin gene expression profiles associated with seasonal variation in migratory state and endurance flight in yellow-rumped warblers (*Setophaga coronata*). Bioinformatics analysis and real-time qPCR were used to analyse the expression of galectins in flight muscle, heart and liver tissues of 15 warblers separated into three groups of winter unflown, and fall migratory flown/unflown birds. Five transcripts similar to chicken and human galectins −1, −2, −3, −4, and −8 were identified in warbler tissues. The expression of these galectins showed no seasonal changes between two experimental groups of birds maintained under unflown winter and fall conditions indicating a minor role of galectins in preparation for migration. However, endurance flight led to a significant elevation of galectin-1 and galectin-3 mRNAs in flight muscles and galectin-3 mRNA in heart tissue while no changes were observed in liver. Different changes were observed for the level of *O*-GlcNAcylated proteins, which were elevated in flight muscles under winter conditions. These results suggest that secreted galectin-1 and galectin-3 may be active in repair of bird muscles during and following migratory flight and serve as molecular biomarkers of recent arrival from migratory flights in field studies.

Keywords Galectin · O-GlcNAc · Bird migration · Flight · Exercise · Muscle · PCR

Introduction

Migration in birds typically involves the regulated seasonal movement between breeding and wintering grounds. Migration is energetically costly, requiring intensive exercise for long periods of time, with feeding and refueling occurring at stopover sites. Since migrants must rely solely on stored energy and nutrients for flight, there are changes in body composition, metabolism, and enzyme activity during migratory season [1]. Endurance flight requires a high sustained aerobic capacity, along with other physiological adjustments to flight muscle to support the demands of prolonged exercise [2]. Flight has been demonstrated to affect bacterial killing ability and constitutive components of innate immune function in western sandpipers (*Calidris mauri*) and European starlings (*Sturnus vulgaris*) [3, 4]. Tradeoffs between the energy allocated to flight versus other biological processes may be evident in changes in gene expression that result from the physiological stress associated with muscle damage, muscle catabolism, oxidative stress, inflammation, and immune changes – all occurring during migration and migratory flight [1, 5, 6].

Galectins are multifunctional soluble β-galactoside-binding proteins capable of modulating cell differentiation, proliferation, survival, death, adhesion, and migration [7]. These cellular events are critical in biological processes such as embryogenesis, angiogenesis, neurogenesis, tissue regeneration, and immunity. Sixteen galectin genes have been

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described in animal kingdom, however, there are variations between species. For instance, twelve galectin genes (*LGALS* 1, 2, 3, 4, 7, 8, 9, 10, 12, 13, 14, 16) are expressed in human tissues [8], while only five galectins (CG-1A, CG-1B, CG-2, CG-3, and CG-8) are known in chicken tissues [9]. Galectins are classified into three subfamilies depending on the architecture of carbohydrate-recognizing domains (CRDs). Proto-type galectins (−1, −2, −5, −7, −10, −11, −13, −14, −15, and −16) possess a single CRD and usually form homodimers, tandem-repeat galectins (−4, −6, −8, −9, and −12) contain two different covalently-linked CRDs, and chimera-type galectin-3 carries one CRD with an extended non-lectin N-terminal domain essential for oligomerization [7, 8, 10]. The expression patterns of galectin genes and proteins change substantially in cells exposed to stress such as hypoxia, oxidative stress, and ultraviolet light as well as under processes associated with tumorigenesis and inflammation, which implicate galectins as potential regulators and biomarkers for cellular stress responses [8]. The role of galectins as cellular stress regulators is supported by their ability to modulate both pro-survival and pro-apoptotic pathways [11], the balance of which is essential for variety of physiological functions including those associated with physical activity and tissue development and regeneration. For instance, galectin-1 is required for skeletal muscle regeneration [12, 13]. This galectin is found within striated muscle tissues in association with sarcomeric actin on I bands [14] and also co-localizes with actin in human platelets [15]. Galectin-3 is a reliable biomarker of heart failure and myocardial fibrosis, which can be assessed in plasma and blood [16]. Serum concentration of galectin-3 significantly increases in healthy marathon runners after endurance training [17, 18]. There is evidence of galectin-2 and galectin-8 association with functions of smooth, skeletal, and cardiac muscles in different organisms [19, 20].

During bird migration, moderate muscle damage has been shown to occur in flight muscle of western sandpipers (*Calidris mauri*) and bar-tailed godwits (*Limosa lapponica*) [6]. Skeletal muscle is partially catabolized to act as a source of protein during long-distance migration [5]. Transcriptomic analysis has also shown that there are alterations in signaling pathways related to muscle satellite cell proliferation and differentiation during migration of yellow-rumped warblers (*Setophaga coronata*) as well as upregulation of several mechanisms that are implicated in galectin function such as the TNF signaling, apoptotic and phagocytic pathways [21]. The regulation of these pathways may involve specific glycosylation of intracellular proteins at the hydroxyl group of their serine and threonine residues by *N*-acetylglucosamine termed *O*-GlcNAc modification or *O*-GlcNAcylation [22]. In particular, *O*-GlcNAcylation is a general mechanism associated with changes in nutrient metabolism [23], cellular stress responses [24], and physical exercises [25–28], which control both localization and activity of *O*-GlcNAcylated proteins in cells [29]. Whether this regulation can be applied to galectin functions is unknown, although at least galectin-1 was reported to undergo *O*-GlcNAc modification [30]. The current knowledge about expression and functions of galectins in birds is mostly limited to the domestic chicken (*Gallus gallus*), where galectins CG-1A and CG-8 have been found to be associated with avian limb skeleton development and morphogenesis [31]. Given the multifunctionality of galectins and their mentioned association with muscle tissues, we hypothesized that galectins may change seasonally as birds change between migratory and resident states, and may be affected by acute endurance flight. In the present study, we identified galectin transcripts in tissues of yellow-rumped warblers and investigated the seasonal and flight-related changes in galectin expression profiles in flight muscles, heart, and liver. We demonstrate the potential significance of galectins as molecular biomarkers of bird migration and recent arrival for field studies in the context of persistent muscular load.

Materials and methods

Birds

Fifteen juvenile male yellow-rumped warblers (*Setophaga coronata*) were captured at Long Point, Ontario in September 2012, and housed at the Advanced Facility for Avian Research under a scientific collection permit from the Canadian Wildlife Service (CA0256), and an animal ethics protocol from the University of Western Ontario Animal Use Sub-Committee (#2010–216). The birds were kept at 21 °C on a natural fall photoperiod (12 h L: 12 h D) to maintain the fall migratory state. For the migratory flown treatment (MF group) 5 birds were flown individually at night for four hours in a wind tunnel designed for avian flight studies (8 m/s, 15 °C, 4 h flight ~115 km) to simulate an endurance flight. Five birds were sampled at the time of lights-off for the migratory unflown treatment (MU group). After the flight experiments were complete, 5 birds were kept under shorter daylight hours to induce a non-migratory, winter condition (9 h L: 15 h D). After 60 days on the winter photoperiod, these birds were sampled at lights-off for the winter unflown treatment (WU group). Immediately after flight or at lights-off birds were killed by decapitation under isoflurane anaesthesia so that tissue samples could be snap-frozen with liquid nitrogen, and stored at -80 °C.

Validation of galectin transcripts and PCR primers design

A *de novo* muscle transcriptome from the warblers was assembled using Trinity Software, and submitted to the KEGG to obtain gene identifiers [21]. The gene identifiers were screened for galectin genes versus chicken galectins, which generated the potential avian galectin transcript sequences

used in this study (Fig. S1, Supplementary Materials). The validation of LGALS4 sequence was based on human *LGALS4* since a chicken homolog is not available. The alignment of galectin transcripts to the coding DNA sequences (CDSs) of known human and chicken galectins was performed using the Clustal Omega tool (http://www.ebi.ac.uk/Tools/msa/clustalo/). The validated sequences were used to design oligonucleotide primers for LGALS1, LGALS2, LGALS3, LGALS4, and LGALS8 using the Primer-BLAST online software [32] for conventional and real-time PCR (Table 1). The primers were ordered from BioCorp UWO OligoFactory (Department of Biochemistry, The University of Western Ontario).

RNA isolation and cDNA synthesis

Total RNA was extracted from 50 to 100 mg of frozen tissues using TRIzol reagent (Ambion, Life Technologies) and glass homogenizer as per the manufacturer protocol and quantified with a NanoDrop 2000C spectrophotometer. The 260 nm/280 nm ratio of all purified RNA samples was ~1.9 and RNA integrity was confirmed by agarose gel electrophoresis in the presence of ethidum bromide by checking the 18S and 28S ribosomal RNA bands. cDNA synthesis was carried out in 20 μl reaction volume containing 1 μg of RNA and all required components of the Maxima First Strand cDNA Synthesis Kit for RT-qPCR (Thermo Scientific, cat. #K1642).

Conventional end-point PCR, gel electrophoresis, and sequencing of PCR amplicons

Conventional PCR and DNA sequencing were done to ensure the quality of primers and identity PCR amplicons. The end-point PCR was performed in T100™ Thermal Cycler (BioRad) in a 50 μl reaction mix containing 1 μl of original cDNA and all components of the 2X Taq FroggaMix (FroggaBio, cat. #FBTAQM) using the following regime: an initial denaturation step of 3 min at 94 °C followed by 35 cycles at 94 °C for 30 s, 56 °C for 30 s, and 72 °C for 60 s with the final step of 5 min at 72 °C. PCR amplicons were separated by electrophoresis on a 2% agarose gel in the presence of SYBR® Safe DNA Gel Stain (Invitrogen) and the gels were viewed and imaged using a Molecular Imager GelDoc XRT (BioRad). The single bands with confirmed sizes were excised; PCR amplicons were purified using a gel/PCR DNA Fragments Extraction Kit (Geneaid, cat. #DF100) and sequenced at the DNA Sequencing Facility (Robarts Research Institute, London, Ontario).

Real-time quantitative PCR (qPCR)

qPCR was performed in a Rotor-Gene 6000 Real-Time Rotary Thermocycler (Corbett Life Science, Concorde, NSW, Australia) using SensiFAST SYBR No-ROX Kit (Bioline, #BIO-98002) as per the manufacturer protocol. The reaction volume was 20 μl with 1 μl of a 10-fold diluted cDNA and three technical replicates were used for each sample. The expression of galectin genes was quantified by the ΔΔCt method using GAPDH (glyceraldehyde 3-phosphatedehydrogenase) as a housekeeping gene and shown as a fold change to MU samples. We were unable to validate a stable housekeeping gene for heart that could include the winter samples and so no seasonal comparisons were made for heart.

Tissue lysate preparation and protein quantification

Frozen flight muscle tissue samples were weighed (6 to 47 mg range) and lysed with RIPA buffer (10 mM Tris•HCl, pH 8, 1% Triton X-100, 0.1% SDS, 0.5 mM EGTA, 0.1% sodium deoxycholate, 140 mM NaCl) containing six protease/phosphatase inhibitors (1 mM PMSF, 1 mM Na_3VO_4, 1 mM AEBSF, 5 mM EDTA, 50 μM leupeptin and 1 μM pepstatin) at 1:19 ratio (mg tissue: ml buffer) on ice. Briefly, tissue samples were minced with small scissors, ground with a plastic

Table 1 The list and properties of oligonucleotide primers for qPCR designed to detect the expression of specific transcripts in yellow-rumped warblers (*Setophaga coronata*) tissues

Gene symbol	Transcript ID	Primer sequence, 5′- > 3′ (**F**orward, **R**everse)	Annealing positions	Amplicon size, bp
LGALS1	comp39822_c0_seq1	**F** GGATGTTGAGCCTGGTGAGT	59–78	125
		R AGTCAAAGCGAGGGTTGAAA	164–183	
LGALS2	comp95053_c0_seq7	**F** TCCAAGTGCTCCGATTCCTG	919–938	173
		R TAGGTGATGTTGCGGTAGCC	1091–1072	
LGALS3	comp83947_c0_seq1	**F** CCCAGGACAACCATCCAGTG	418–437	197
		R TTGGGTTGGGGTTCACAGTC	614–595	
LGALS4	comp94371_c1_seq4	**F** CTTCAAGGTGTTTGCCGAGG	1108–1127	109
		R GTAGGACAGGACCACATCGC	1216–1197	
LGALS8	comp97633_c1_seq1	**F** GCACTTGCTGCTCTACAACC	590–609	124
		R CGATGGCTGAGAGCCTTGTA	713–694	
GAPDH	comp90806_c0_seq4	**F** TCCCGAAGCGGTAAAGATGG	18–37	181
		R CCGGAAGTGGCCATGAGTAG	198–179	

pestle in 1.5 ml Eppendorf tubes, and sonicated 3 × 25 sec with a MicrosonTM XL-2000 Ultrasonic Liquid Processor (Qsonica). The homogenates were centrifuged at 15,000 g for 15 min, 4 °C and the supernatants were used for further analysis. Protein concentration was determined using the *DC*™ Protein Assay Kit from Bio-Rad as per the manufacturer protocol for microplates.

Immunodot blot assay for global *O*-GlcNAc

Proteins (4 μg per well, 6 technical replicates) were immobilized onto a nitrocellulose membrane (GE Healthcare, cat. #10401396) using the Bio-Dot® Microfiltration Apparatus (Bio-Rad) as per the manufacturer protocol. The membrane was exposed consequentially overnight at 4 °C to mouse monoclonal pan-specific *O*-linked *N*-acetylglucosamine primary antibody RL2 (ThermoScientific, cat. #MA1-072, dilution 1:1000) and for 1 h at room temperature to horseradish peroxidase-conjugated goat anti-mouse secondary antibody (Santa Cruz Biotechnology, cat. #sc-2005, dilution 1:10,000). Luminata™ Forte Western HRP Substrate (Millipore) was used for chemiluminescence detection of immunodots, which were visualized in the ChemiDoc® XRS system (Bio-Rad) and quantified as per Dot Blot Analysis protocol of ImageJ (Rasband, W.S., ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA, https://imagej.nih.gov/ij/, 1997–2016).

Statistical analysis

Data were analyzed with SAS® version 9.4 (SAS Institute INC., Cary, NC) with t-tests because we were specifically interested in examining seasonal change (MU versus WU) and the acute response to flight (MU versus MF). Muscle and heart galectin expression was log transformed to achieve a normal distribution, and untransformed values are reported. All results are presented as means ± SEM, $n = 5$ per group except for MF muscle and MU heart groups where we were unable to isolate high quality RNA from one individual and as a result $n = 4$. Pearson correlation was used to compare galectin expression within tissues. Statistical significance was set at $p < 0.05$.

Results

To study the expression of galectin transcripts in tissues of yellow-rumped warblers, we designed appropriate primers for qPCR based on the flight muscle transcriptome (Table 1). BLAST analysis of these oligonucleotides revealed a 100% identity within many predicted and confirmed galectin mRNA regions of other bird species such as finches, crows, sparrows, cranes, flycatchers, and others (Table S1, Supplementary Materials). These results indicate a high level of conservation between avian galectins at the genetic level and validate a broader usefulness of the select galectin primers for various bird species.

Bioinformatics analysis of whole tentative galectin transcripts from the *de novo* muscle transcriptome of warblers revealed a relatively high level of homology (>70% identity scores) with the CDSs of chicken galectin genes (Table 2). Remarkably, the high identity score of 88.15% was noticed for galectin-1 warbler transcript and chicken galectin CG-16 or CG-1A, which represents an isoform of galectin-1 expressed in embryonic thigh/pectoral muscles [33, 34]. The homology of warbler galectin transcripts with human galectins was lower and scored at approximately 60%. Although no chicken galectin-4 was reported in literature, the tentative warbler galectin-4 transcript also showed strong homology (69% query cover by nucleotide BLAST) to a predicted avian galectin-4 mRNA from ground tits (*Pseudopodoces humilis*) (XM_014262618.1).

All five galectin transcripts (LGALS1, LGALS2, LGALS3, LGALS4, and LGALS8) were readily detected by qPCR in flight muscle tissues of yellow-rumped warblers and their identity was confirmed by agarose gel electrophoresis (Fig. 1) and DNA sequencing analysis (data not show). In flight muscle (Fig. 2a), there was no significant seasonal effect on the expression of all galectins or flight related variation for galectins −2, −4 or −8 (all $t < 1.55$, $P > 0.164$). However, LGALS3 significantly increased ($t = 2.57$ $P = 0.037$) and LGALS1 tended to increase ($t = 1.93$, $P = 0.095$) during flight. Since muscle LGALS1 did not differ between WU and MU samples we combined them for comparison to flown birds. With this larger sample size LGALS1 was significantly elevated during flight ($t = 2.30$, $P = 0.040$). In the heart (Fig. 2b) LGALS1, LGALS2, LGALS3, LGALS4, and LGALS8 expression was not affected by flight (all $t < 1.49$, $P > 0.181$), but LGALS3 increased during flight ($t = 3.02$, $P = 0.020$). No galectin transcripts varied between seasons or with flight in liver (Fig. 2c; all $t < 1.64$, $P > 0.140$).

To determine potential co-regulation among galectin types we explored the correlations among galectin expression within individuals (Tables S2-S4, Supplementary Materials). Only three significant relationships emerged (Fig. 3). In the muscle LGALS1 was positively related to LGALS4 ($r = 0.684$, $P = 0.007$). In the liver LGALS4 was positively related to LGALS1 ($r = 0.644$, $P = 0.010$) and to LGALS2 ($r = 0.577$, $P = 0.024$).

Lastly, we assessed the level of *O*-GlcNAc in flight muscles as a cellular sensor for metabolic status associated with different types of physical load and nutrient deficiency [23, 28, 35]. Immunodot blot assay revealed that there was a significant ($P = 0.022$) increase in the *O*-GlcNAc level in WU samples versus both MF and MU samples while no differences were detected between flown and unflown fall

Table 2 Percent identity scores (Clustal Omega alignment) of warbler galectin transcripts versus the coding DNA sequences of chicken and human galectins. NCBI reference sequences or transcript identifiers are specified in brackets

Galectin	*Setophaga coronata*	*Gallus gallus*	*Homo sapiens*
LGALS1	100% (K06830)	58.33% (NM_205495.1)	64.94% (NM_002305.3)
		88.15% (NM_206905.1)*	63.25% (NM_002305.3)
LGALS2	100% (K10090)	73.13% (XM_015288417.1)	58.65% (NM_006498.2)
LGALS3	100% (K06831)	72.55% (NM_001302800.1)	61.84% (NM_002306.3)
LGALS4	100% (K10091)	Not available	60.28% (NM_006149.3)
LGALS8	100% (K06832)	73.70% (NM_001010843.1)	56.24% (NM_201543.2)

*Chicken LGALS1 paralog known as CG-16 (CG-1A)

conditions (Fig. 4). These observations suggest that in contrast to galectin expression, *O*-GlcNAcylation in bird tissues is regulated in a circadian- or seasonal (winter versus fall conditions)- dependent manner.

Discussion

Galectins comprise a complex network of soluble proteins, which are found both outside cells and in different subcellular compartments (cytosol, mitochondria, and nucleus), where they regulate diverse cellular processes including, but not limited to cell growth, survival, apoptosis, cell migration, adhesion, signaling, and pre-mRNA splicing [7, 8]. Different galectin family members also show differential expression among tissues, highlighting an additional level of their regulatory capacity [36]. As a comparative reference, human muscle tissues express high mRNA and protein levels of galectin-1, galectin-3, and galectin-8 and very low or negligible levels of galectin-2 and galectin-4. Functions and tissue-specific distribution of galectins in avian tissues are relatively less studied except for *Gallus gallus* as a model system [9, 31], which has inherent limitations and does not serve as a good model to study ecophysiological and biochemical aspects of migration in birds. Our study revealed expression of five galectin transcripts in flight muscles, heart, and liver, which have a high homology with chicken LGALS1, LGALS2, LGALS3, LGALS8 and human LGALS4. Overall, we noticed no

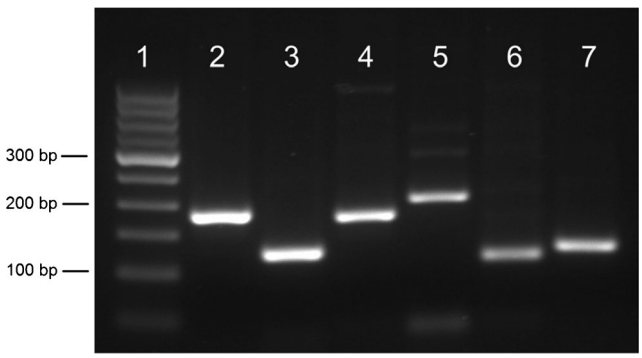

Fig. 1 End-point RT-PCR detection of galectin transcripts in muscle tissues of yellow-rumped warblers (*Setophaga coronata*). All PCR amplicons show expected sizes: 1, DNA ladder (50–500 bp Low Range DNA Marker, Bio Basic, cat. # GM305), 2 – GAPDH (181 bp), 3 – LGALS1 (125 bp), 4 – LGALS2 (173 bp), 5 – LGALS3 (197 bp), 6 – LGALS4 (109 bp), and 7 – LGALS8 (124 bp)

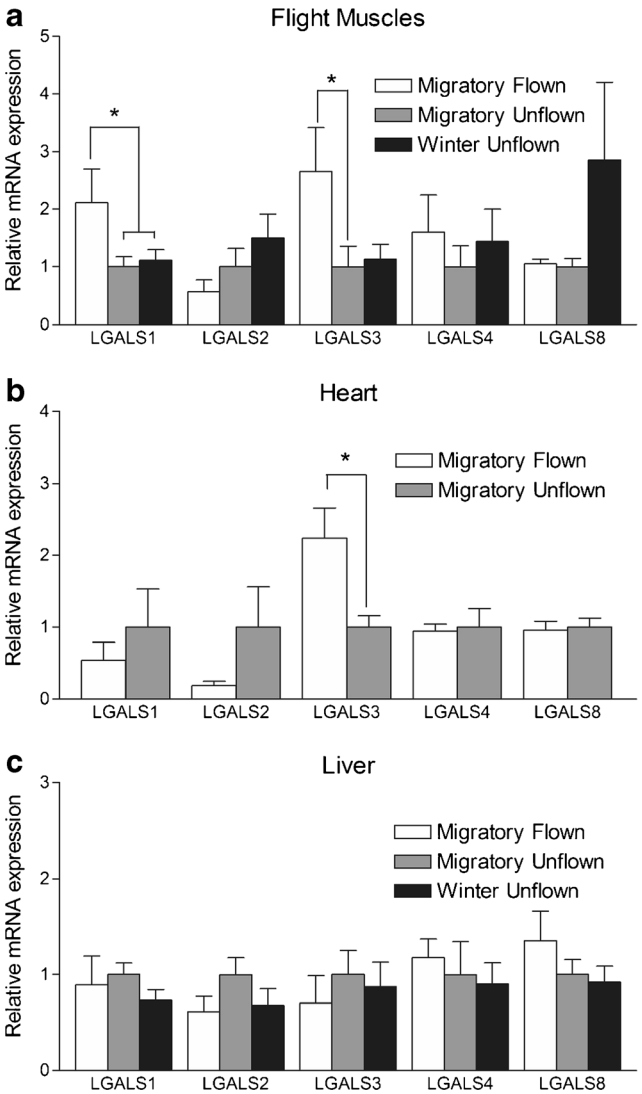

Fig. 2 Differential expression of galectin transcripts in three different tissues of yellow-rumped warblers (*Setophaga coronata*). The data are presented as ΔΔCt values calculated for each sample with normalization to GAPDH as a housekeeping gene and to the galectin gene expression in the migratory unflown group of birds. Bars show means ± SEM of 4–5 biological replicates. *$P < 0.05$, Student's t-test, two-tailed

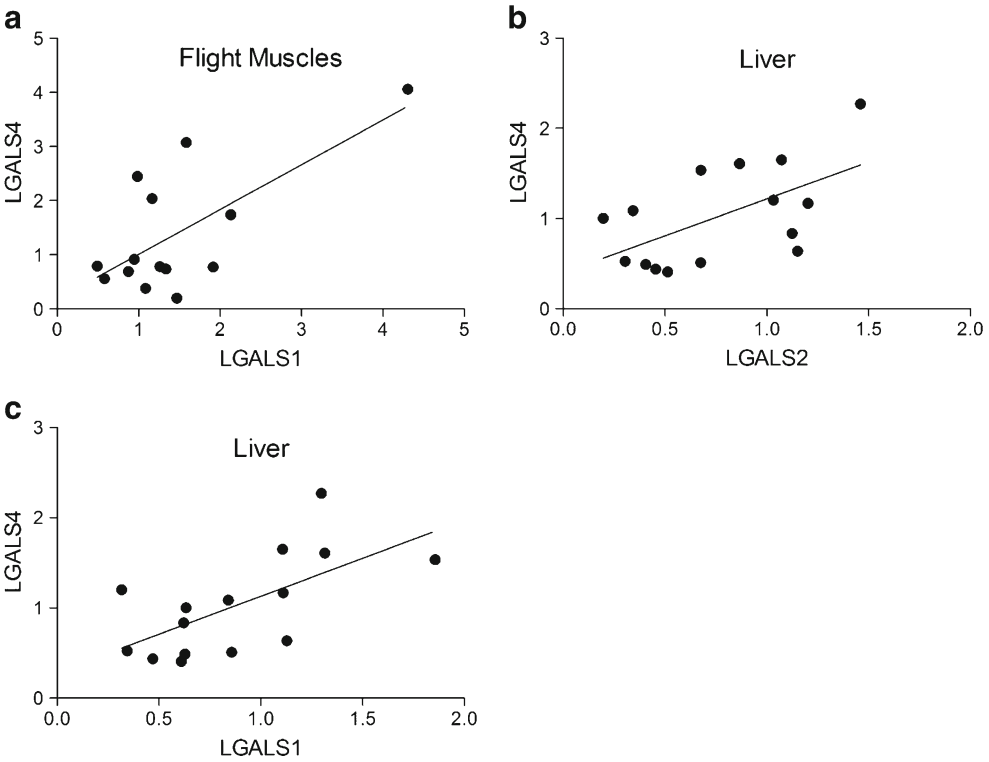

Fig. 3 Significant correlations between galectin gene expression in bird tissues. The data present all measurements of LGALS1 versus LGALS4 ($n = 14$) in flight muscles and LGALS1 and LGALS2 versus LGALS4 in liver ($n = 15$) regardless of seasonal or migratory status

seasonal differences (winter versus migratory unflown groups) in the expression of these galectins in all studied tissues, which indicates no anticipatory up- or down-regulation of galectins in preparation for migration. However, we observed elevation of LGALS1 and LGALS3 in muscle tissues with flight, showing an acute response which may be involved in repair processes after a bird arrives at stopover. Galectins −2, −4, and −8 mRNAs showed a stable expression regardless of the tissue tested (flight muscle, heart, or liver). These findings suggest that regulation and functions of different galectins in migration of birds are complex and that only specific galectins show flight-dependent changes associated with muscle tissues.

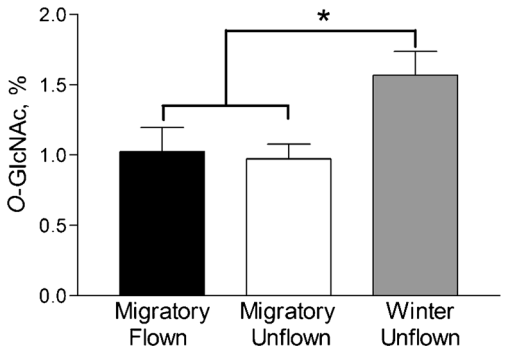

Fig. 4 Global *O*-GlcNAc levels in flight muscle tissues of yellow-rumped warblers (*Setophaga coronata*). The data present the integrated density of 84 immunodots (6 technical replicates of 14 tissue samples) as a percentage of the total measured using the gel analysis procedure of ImageJ (rsb.info.nih.gov/ij/docs/menus/analyze.html#gels). Bars show means ± SEM of 4–5 biological replicates. *$P = 0.022$, Student's t-test, two-tailed

The biological significance of galectin-1 in the context of muscle activity including endurance flight seems to be very relevant since we noticed a strong increasing trend in the expression of this transcript in flight muscle. Indeed, galectin-1 is an actin-binding protein [15] strongly associated with striated muscles [14]. An increase in galectin-1 mRNA expression may result from the flight-associated damage of myocytes and consequent compensatory upregulation of this gene required for efficient muscle activity. Moreover, galectin-1 can directly facilitate muscle regeneration [13, 37], induce the differentiation of human mesenchymal stem cells into skeletal muscle cells [12, 38], and improves muscle functions in mice models [39]. These functions of galectin-1 seem to be less relevant to cardiac muscles in birds and certainly not attributed to liver cells, although an increased level of galectin-1 was reported in mice heart tissues in association with acute myocardial infarction [40]. An additional outcome of galectin-1 upregulation in migration of birds could be an involvement in acute inflammation and immune responses helping birds to resist immunodeficiency at stopover.

Upregulation of LGALS3 in flight and cardiac muscles of migrating birds is consistent with a well-known phenomenon of galectin-3 accumulation in serum of healthy subjects performing intensive physical exercises such as marathon runners [17, 18]. Endurance flight and marathon running represent similar increases in physical load over a prolonged time and galectin-3 can be considered as a molecular biomarker of

this physical challenge at both transcript and protein levels. Notably, galectin-3 is a reliable marker of heart failure and fibrosis [41], which reflect the tissue damage and correlate with accumulation of collagen in myocardium [42–44]. It should be noted that *LGALS3* is a classical stress-sensitive gene, which is upregulated by hypoxia, serum deprivation, oxidants, and other stress stimuli in a variety of human cell culture models [8, 45–47] due to the presence of HIF1α and NF-κB recognizing domains in its promoter [45]. Functions of galectin-3 are largely variable depending on its localization in cells, namely extracellularly galectin-3 induces apoptosis [48] and mediates cell adhesion [49], in the nucleus it regulates pre-mRNA splicing [50] and expression of certain genes [51], and in cytoplasm it inhibits apoptosis [52, 53] and modulates transmembrane signaling [54]. As such, source, amount, and localization of galectin-3 are critical unknowns, which require further attention to understand the physiological significance of galectin-3 upregulation in migration of birds, which might be mostly related to tissue repair mechanisms. Indeed, changes in the expression of galectin-3 (and galectin-1) can be metabolic, since the activity of key metabolic enzymes in the muscles of migrant birds are upregulated, compared to non-migratory birds [55]. In the autumn, flight muscles are primed by promoting protein-mediated fatty acid transport and oxidation [56]. In terms of molecular mechanisms, flight-induced oxidative stress in bird tissues [21] could be responsible for the upregulation of LGALS1 and LGALS3 as recently demonstrated in a model of granulocyte differentiation of human HL-60 cells [47].

The mRNA expression of galectins −2, −4, and −8 in skeletal muscles, heart, and liver was not changed regardless seasonal or flight-related variations. These specific galectins are relatively less studied than galectins −1 and −3, and it is premature to explain their stable expression between bird tissues. Most likely, these galectins fit to the category of microenvironmental stress-resistant galectins [8] balancing a physiological sustainability of galectin networks in different tissues of healthy migrant birds. It should be noted, however, that the expression of galectins −2, −4, and −8 is misbalanced and significantly increased in sera of patients with colorectal and breast cancer [57] demonstrating challenging differences of physiological and pathological galectin networks. As we noticed significant positive correlations in the expression of galectin-1 transcripts versus galectin-2 and galectin-4, further thorough examination of these galectins is required using an increased sample size of birds. Molecular mechanisms responsible for differential expression and localization of galectins in tissues from flown and unflown birds remain to be studied. We can suggest, however, that the migratory phenotype requires secreted rather than intracellular galectins because the level of *O*-GlcNAc was decreased in flight muscles of birds from this group. Indeed, *O*-GlcNAcylation is an intracellular process and none of *O*-GlcNAcylated proteins reside on the outside of cells [22]. Therefore, it is likely that fall migratory conditions shift the *O*-GlcNAc dynamic balance to hypoglycosylation in comparison with winter conditions as per circadian rhythm regulation [58, 59]. The lower *O*-GlcNAc levels may provide a better setup and readiness for endurance flight, although it could vary depending on the biological context of physical exercises. For instance, protein *O*-GlcNAcylation was decreased in heart of swim-exercised mice [25] while it was increased in rat skeletal muscles as a result of treadmill running [27, 28]. Many variables such as exercise load, time, tissue type, and circadian rhythm should be considered in the future to explain these differences.

In conclusion, this study characterized for the first time the galectin mRNA expression profiles in flight muscle, cardiac, and liver tissues of yellow-rumped warblers, a non-model organism used to study ecophysiology and biochemistry of bird migration. Transcripts of five galectins were identified in all tissues, and in response to a four-hour endurance flight galectin-3 was found to be upregulated in both flight muscles and heart tissue whereas galectin-1 was upregulated in flight muscles only. These galectins are good candidates as molecular biomarkers of bird migration and recent arrival for field studies, although a functional significance of the galectin upregulation remains to be discovered. Since there is evidence of galectin activity being indicative of acute exercise in flight and heart muscles and decreased level of *O*-GlcNAc in flight muscle, it could be advantageous to expand this study to measure circulating levels of galectin proteins in blood.

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Compliance with ethical standards

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Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

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