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Context-dependent regulation of pectoralis myostatin and lipid transporters by temperature and photoperiod in dark-eyed juncos

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Abstract

A prominent example of seasonal phenotypic flexibility is the winter increase in thermogenic capacity (=summit metabolism, $\dot{M}_{sum}$) in small birds, which is often accompanied by increases in pectoralis muscle mass and lipid catabolic capacity. Temperature or photoperiod may be drivers of the winter phenotype, but their relative impacts on muscle remodeling or lipid transport pathways are little known. We examined photoperiod and temperature effects on pectoralis muscle expression of myostatin, a muscle growth inhibitor, and its tolloid-like protein activators (TLL-1 and TLL-2), and sarcolemmal and intracellular lipid transporters in dark-eyed juncos *Junco hyemalis*. We acclimated winter juncos to four temperature (3 °C or 24 °C) and photoperiod [short-day (SD) = 8L:16D; long-day (LD) = 16L:8D] treatments. We found that *myostatin*, *TLL-1*, *TLL-2*, and lipid transporter mRNA expression and myostatin protein expression did not differ among treatments, but treatments interacted to influence lipid transporter protein expression. Fatty acid translocase (FAT/CD36) levels were higher for cold SD than for other treatments. Membrane-bound fatty acid binding protein (FABPpm) levels, however, were higher for the cold LD treatment than for cold SD and warm LD treatments. Cytosolic fatty acid binding protein ($FABP_c$) levels were higher on LD than on SD at 3 °C, but higher on SD than on LD at 24 °C. Cold temperature groups showed upregulation of these lipid transporters, which could contribute to elevated M_{sum} compared to warm groups on the same photoperiod. However, interactions of temperature or photoperiod effects on muscle remodeling and lipid transport pathways suggest that these effects are context-dependent.

Key words: birds, FABPpm, FABPc, FAT/CD36, myostatin, pectoralis, phenotypic flexibility, photoperiod, temperature.

Seasonal phenotypes in small birds are one well-known example of reversible phenotypic flexibility and allow birds to better match their phenotypes to seasonal climates (Swanson 2010; Swanson and Vézina 2015). Small birds experience high thermoregulatory demands in cold climates, and due to their high surface area-to-volume ratios, they are expected to undergo substantial variation in metabolic phenotypes between seasons. The winter phenotype in small birds is distinguished by improved cold tolerance and elevated basal (BMR; minimum maintenance metabolism) and summit (M_{sum}; maximum cold-induced metabolism) metabolic rates relative to summer (Dubois et al. 2016). Such increments of metabolic rates, especially M_{sum}, are positively correlated with cold tolerance (Swanson, 2001; Swanson and Liknes 2006). As a result, M_{sum} is commonly interpreted as an indicator of a bird's ability to endure cold environments.

Adjustments in M_{sum} throughout the annual cycle in birds may be mediated through variation in skeletal muscle (Marsh 1981; Evans et al. 1992; Liknes and Swanson 2011b) and heart (Piersma

1998; Battley et al. 2001; Swanson et al. 2014b) masses and/or cellular metabolic intensities (Liknes and Swanson 2011a; Zheng et al. 2014) coupled with enhanced substrate transport (Liknes et al. 2014a). Previous studies indicated that flight muscle hypertrophy contributed to increases in organismal metabolic rates (Marsh 1981, 1984; Evans and Rose 1988; Dietz et al. 1999; Petit and Vézina 2014), especially for winter phenotypes in birds (Swanson et al. 2009, 2013; Liknes and Swanson 2011b; Petit et al. 2014). Because seasonal increases in pectoralis muscle and heart masses consistently contribute to the winter phenotype in small birds, the mechanisms regulating seasonal muscle remodeling are an important research target for understanding the flexible metabolic phenotypes in birds. One candidate for the flexible regulation of skeletal muscle mass is myostatin, which belongs to the TGF-β family of growth factors and is an autocrine/paracrine inhibitor of muscle growth in birds and mammals (Lee 2004). Myostatin acts to inhibit skeletal muscle growth and reduces muscle mass in birds and mammals, thereby regulating muscle remodeling in adults (Lee 2004; Rodgers and Garikipati 2008). Several studies have examined variation in expression of myostatin in birds during migration and winter acclimatization accompanied by variation in muscle mass (Swanson et al. 2009, 2014a; King et al. 2015). These studies offer variable support for a role for myostatin in regulating flexible muscle masses throughout the annual cycle in birds, with some finding reduced expression of either mRNA or protein for myostatin in pectoralis muscle (Swanson et al. 2009), or no changes in other species for migratory or wintering phenotypes (Swanson et al. 2014a; King et al. 2015). However, white-throated sparrows *Zonotrichia albicollis* under migratory photoperiod exhibited the opposite effect, with elevated mRNA expression of *myostatin* compared to the winter photoperiod, but European starlings *Sturnus vulgaris* did not show any changes in *myostatin* mRNA expression with exercise training (Price et al. 2011). In contrast, exercise- and cold-trained house sparrows *Passer domesticus* showed significantly reduced myostatin protein levels associated with increased pectoralis mass compared to their controls (Zhang et al. 2015b).

Myostatin is secreted as an inactive form and requires cleavage by metalloproteinases, including the tolloid-like proteins TLL-1 and TLL-2, to form the active C-terminal dimer myostatin (Huet et al. 2001). Thus, upstream of myostatin receptors, regulation of muscle mass through myostatin can involve both synthesis of myostatin and activation of the protein via cleavage (Lee 2004, 2008). *Myostatin* and *TLL-1* gene expression were both downregulated in winter house sparrows relative to summer sparrows (Swanson et al. 2009). Moreover, mRNA expression for either or both *TLL-1* and *TLL-2* were downregulated in winter relative to summer for American goldfinches (*Spinus tristis*) and black-capped chickadees (*Poecile atricapillus*, Swanson et al. 2014a). In contrast, Price et al. (2011) found that *TLL-1* mRNA expression did not differ from controls for exercise-trained European starlings. Moreover, exercise- and cold-trained house sparrows showed only non-significant variation in TLL mRNA expression in trained groups compared to their controls (Zhang et al. 2015b). Thus, although some evidence suggests that modulation of myostatin and myostatin processing capacity may regulate phenotypic flexibility of metabolic capacities in birds in response to changing energy demands, this modulation does not appear to be universal for birds under all such conditions.

In addition to flight muscle hypertrophy, winter and migratory enhancement of lipid transport (Liknes et al. 2014; Zhang et al. 2015d) and catabolism (Marsh and Dawson 1989; Liknes and Swanson 2011a; Corder et al. 2016) also occurs in birds. Prolonged shivering thermogenesis in birds is almost exclusively fueled by exogenous lipids from adipose tissues (Swanson 2010). As a result, capacities for non-esterified fatty acid (NEFA) uptake into the myocyte and their subsequent transport to mitochondrial membranes are potential regulatory steps for lipid catabolic capacity in support of shivering (Swanson 2010; Zhang et al. 2015a). Once circulating NEFAs reach the myocyte, plasma membrane-bound fatty acid binding protein (FABPpm) and fatty acid translocase (FAT/CD36) cooperate to transport NEFAs across the endothelium, the interstitial space, and the sarcolemma to enter muscle cells (Kiens 2006; Glatz et al. 2010). The effects of winter acclimatization or migration on skeletal muscle FAT/CD36 and FABPpm levels are incompletely resolved. Some studies showed elevated FAT/CD36 and FABPpm levels during migration compared to non-migrants (McFarlan et al. 2009; Zhang et al. 2015c), but others indicated stable FAT/CD36 and FABPpm during migration or wintering (Zhang et al. 2015c, 2015d). Moreover, results from exercise and cold training studies suggest that responses of FAT/CD36 and/or FABPpm levels to increasing energy demands also vary among species (Price et al. 2010; Zhang et al. 2015a). Intramyocyte fatty acid transport in skeletal muscles is mediated by cytosolic fatty acid binding protein ($FABP_c$) (Kiens 2006; Guglielmo 2010). $FABP_c$ also acts as an intramyocyte fatty acid receptor, acting to regulate and potentially limit fatty acid uptake (Glatz et al. 2010). Most studies of migration (Guglielmo et al. 2002; McFarlan et al. 2009), cold acclimation (Stager et al. 2015), or winter acclimatization (Liknes et al. 2014; Zhang et al. 2015d) show that increases in pectoralis $FABP_c$ levels are consistent correlates of enhanced flight and shivering performance in birds.

The regulation of winter phenotypes in small resident birds in temperate-zone climates could potentially be driven by environmental cues such as photoperiod (Carey and Dawson 1999) and temperature (Swanson and Olmstead 1999). To examine mechanistic bases for photoperiod and temperature-induced variation in organismal metabolic capacities, we studied dark-eyed juncos *Junco hyemalis* exposed to different photoperiod [long-day (LD) and short-day (SD)] and temperature (cold and warm) treatments. Dark-eyed juncos are a common winter resident in South Dakota and resident populations in western North America show seasonal variation in pectoralis mass and M_{sum} (Swanson 1990, 1991). Moreover, a previous study using the same individual birds indicated that cold exposure, but not short photoperiod, elevated M_{sum} compared to warm exposed birds (Swanson et al. 2014c). This study also demonstrated that even though pectoralis muscle mass did not vary significantly among acclimation treatments, cellular metabolic intensity in the pectoralis was generally higher for LD and cold groups (Swanson et al. 2014c). In addition, Stager et al. (2015) conducted a study of genome-wide transcriptional profiles on pectoralis muscle samples from the same individual birds and found that mRNA expression for muscle hypertrophy, angiogenesis and lipid transport and oxidation pathways were generally upregulated by cold exposure. Several studies, however, have documented that mRNA and protein expression in pectoralis muscle during migration and winter acclimatization in small birds are sometimes not correlated for myostatin or lipid transport pathways (e.g., Swanson et al. 2014a, 2017; King et al. 2015; Zhang et al. 2015d). Consequently, the present study builds upon these previous studies by evaluating, in pectoralis muscle (the main thermogenic organ in birds), both gene and protein expression for myostatin, gene expression for myostatin activators *TLL-1* and *TLL-2*, and gene and protein expression for the lipid transporters, FABPpm, $FABP_c$ (protein only) and FAT/CD36 for these same individual juncos, as well as by examining correlations

among these factors and with M_{sum} and pectoralis muscle mass. We hypothesize that cold exposure will reduce expression of the myostatin system and increase lipid transport capacities, but that photoperiod will have limited impacts on these systems. These analyses will identify the relative variation in pectoralis gene and protein expression for the myostatin and lipid transport systems in response to temperature and photoperiod in small birds and how such variation might relate to seasonal metabolic flexibility.

Materials and Methods

Bird collection and acclimation treatments

All procedures reported herein were approved by the University of South Dakota Institutional Animal Care and Use Committee (Protocol 79-01-11-14C). Details of bird collection and acclimation treatments are provided in Swanson et al. (2014b). Briefly, dark-eyed juncos were captured near Vermillion, South Dakota (approximately 42° 47' N, 97° W) during mid-December under appropriate state (11-7, 12-2) and federal (MB758442) scientific collecting permits. After capture, birds were individually housed in 59 × 45 × 36 cm stainless-steel cages with controlled temperature (± 2 °C) and photoperiod with *ad libitum* mixed seed, protein supplement (mixture of homogenized dog food and hard-boiled egg with seed) and vitamin-enriched (Wild Harvest Multi-Drops vitamin supplement for all birds, United Pet Group, Inc., Cincinnati, OH, USA) water. Birds were acclimated to captive conditions, at room temperature (23 °C) and natural photoperiod (9 h:15 h, light:dark), for at least two weeks. We randomly assigned birds into four temperature–photoperiod treatments: 24 °C, 8 h:16 h light:dark (warm SD); 24 °C, 16 h:8 h light:dark (warm LD); 3 °C, 8 h:16 h light:dark (cold SD); and 3 °C, 16 h:8 h light:dark (cold LD). We included 12 birds in each treatment and acclimation treatments lasted for 6 weeks.

After the 6-week acclimation treatments, we euthanized birds by cervical dislocation and quickly excised the pectoralis muscles on ice. We weighed the left pectoralis muscle to the nearest 0.1 mg and divided it into two sub-samples, one of which was placed in RNAlater (Ambion, Grand Island, NY, USA) for real-time quantitative reverse transcription PCR (qRT–PCR) and one flash-frozen in liquid nitrogen for Western blot analysis. Both sub-samples were stored frozen at −80 °C until later assays.

Quantitative real-time RT–PCR

We measured pectoralis mRNA expression for *myostatin*, *TLL-1*, *TLL-2*, *FABPpm*, and *FAT/CD36* by qRT–PCR, as described in Swanson et al. (2014a) and Zhang et al. (2015a, 2015b, 2015c, 2015d), using *glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*) as a housekeeping gene. For these assays, we first extracted total RNA with β-mercaptoethanol and the RNeasy Fibrous Tissue Mini kit (QIAGEN, Valencia, CA, USA). We then quantified extracted RNA with an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA) and used 50 ng of purified RNA for qRT–PCR reactions with a TaqMan RNA-to-CT 1-Step Kit (Applied Biosystems, Carlsbad, CA, USA) and a Step-One-Plus Real-Time PCR System (Applied Biosystems). For all genes, we used the custom qRT–PCR probe and primer sets (Applied Biosystems) containing the sequences listed in Swanson et al (2014a, see their Table 1), which were derived from partial cDNA sequences from house sparrows (Swanson et al. 2009; GenBank Accession Numbers KP337454–KP337456). We conducted the qRT–PCR at 48 °C for 15 min, then 95 °C for 10 min, followed by 40 cycles at 95 °C for 15 s and 60 °C for 1 min. We optimized protocols for all six genes to verify efficiencies for these probe and primer sets for dark-eyed juncos. The $FABP_c$ probe and primer sets failed to amplify mRNA using the StepOnePlus Real-Time PCR System, so we were unable to quantify gene expression for $FABP_c$. Slopes and efficiencies for each gene were: *GAPDH* (−3.35, 98.8%), *Myostatin* (−3.5, 93.1%), *TLL-1* (−3.42, 96.1%), *TLL-2* (−3.49, 93.4%), *FABPpm* (−3.45, 94.9%), and *FAT/CD36* (−3.36, 98.4%). We quantified changes in mRNA expression using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001). We used the mean value for all acclimation treatments for each gene as the reference sample and set the value for this reference sample equal to 1. We then normalized mRNA expression to this reference sample to determine relative amounts of mRNA expression (relative to *GAPDH* expression) for all other samples for the same tissue and species (e.g., Zhang et al. 2015a, 2015b, 2015c, 2015d).

Western blots

We measured pectoralis protein levels for myostatin, FAT/CD36, $FABP_c$, and FABPpm using Western blots with glyceraldehyde phosphate dehydrogenase (GAPDH) as a housekeeping protein (Zhang et al. 2015a 2015b, 2015c, 2015d). We were unable to acquire functional antibodies for TLL-1 and TLL-2, so we did not conduct Western blots for TLL-1 and TLL-2. For Western blot assays, we removed pectoralis samples from −80 °C storage and homogenized small samples by sonication on ice with a Cole-Parmer (Chicago, IL, USA) 4710 Series Ultrasonic homogenizer for three 10 s bursts, with 30 s in between bursts to disrupt membranes and separate membrane proteins from phospholipids. The homogenizing buffer contained 50 mM Tris, pH 7; 100 mM NaCl; 2% SDS. We then separated soluble and phospholipid fractions by centrifugation, and collected the soluble fraction for analysis. We determined protein concentrations on the soluble fraction using a modified DC Lowry improved protein assay and we used 10 µg of protein for analysis via sodium dodecyl sulfate–polyacrylamide gel electrophoresis. We ran all samples on NuPAGE® Novex® 4–12% Bis-Tris protein gels with the same random sample included on every gel to serve as a standard for detecting gel-to-gel variation. After transferring proteins onto membranes, we probed blots with antibodies against myostatin (goat polyclonal; R&D Systems, Minneapolis, MN, USA; 1:100 dilution), FAT/CD36 (rabbit polyclonal; Novus Biologicals, Littleton, CO, USA; 1:1,000 dilution), FABPpm (rabbit polyclonal, from Christopher G. Guglielmo; 1:10,000 dilution), $FABP_c$ (rabbit polyclonal, from Christopher G. Guglielmo; 1:8,000 dilution) (McFarlan et al. 2009), and GAPDH (chicken polyclonal; Millipore, Temecula, CA, USA; 1:8,000 dilution). We washed membranes with TBS-T and incubated them with horseradish peroxidase-conjugated secondary antibodies: anti-rabbit (1:1,000; Santa Cruz Biotechnology, Dallas, TX, USA) for FAT/CD36, FABPpm, and $FABP_c$, anti-chicken (1:1,500; Abcam, Cambridge, MA, USA) for GAPDH, and anti-Goat (1:1,000 dilution; Santa Cruz Biotechnology) for myostatin. For analysis, we visualized blots with the ECL Plus Western Blotting Detection System (GE Healthcare, Buckinghamshire, UK) and captured chemiluminescent images with a VersaDoc 3000 Molecular Imager (Bio-Rad, Hercules, CA, USA). We analyzed images with Quantity One software (Bio-Rad Laboratories), normalizing each protein level by dividing by GAPDH protein levels for the same tissue sample (Zhang et al. 2015b). We used these normalized protein levels for subsequent statistical comparisons.

Table 1. Correlations among mass-independent summit metabolic rate residuals (from regressions of metabolic rate versus M_b

Pearson correlation coefficient					
	M_{sum} residual	PEC residual	Myostatin mRNA level	TLL-1 mRNA level	TLL-2 mRNA level	Myostatin protein level
M_{sum} residual	−0.028	−0.201	0297	0.2	0.277
PEC residual		−0.063	0.095	0.06	−0.187
mRNA expression					
Myostain			0.119	0.127	0.044
TLL-1				0.738**	0.375*
TLL-2					0.251

Swanson et al. (2014b), pectoralis muscle mass (PEC), myostatin mRNA level, tolloid-like protein 1 (TLL-1) mRNA level, tolloid-like protein 2 (TLL-2) mRNA level, protein levels of myostatin in pectoralis muscles of dark-eyed junco. $*P < 0.05$; $**P < 0.01$.

Statistical analyses

We present data as means ± *SE*, unless otherwise noted. We compared mean values of mRNA and protein expression among different treatments with two-way ANOVA. If parametric assumptions of normal distribution (Kolmogorov–Smirnov test) or homogenous variances (Levene's test) were violated, we $\log_{10}$-transformed data prior to comparisons. If significant differences were detected by two-way ANOVA, we used Tukey tests to identify which means differed significantly. We further calculated Pearson correlation coefficients to examine relationships among separate components for each pathway. We obtained values for M_{sum}, body mass (M_b), pectoralis mass, and pectoralis activities of carnitine palmitoyl transferase (CPT, an indicator of fatty acid transport across the mitochondrial membrane), citrate synthase (CS, a key regulatory enzyme of the Krebs cycle), and β-hydroxyacyl Co-A dehydrogenase (HOAD, a key enzyme regulating fat oxidation capacity) for the same individual birds from Swanson et al. (2014c). We tested for correlations for M_{sum}, body mass (M_b), and pectoralis mass with myostatin mRNA and protein expression, and with mRNA expression for the TLLs to examine the muscle remodeling pathway. We tested for correlations for M_{sum} and enzyme activities with fatty acid transporter mRNA and protein expression to examine lipid transport and metabolism pathways. To remove the effects of M_b from analyses of relationships for pectoralis mass and M_{sum}, we calculated residuals from allometric regressions with M_b, and then used least squares linear regression of residuals to test for correlations with these variables. All statistical analyses were conducted with SigmaStat Version 3.5 (Systat, Point Richmond, CA, USA). We accepted statistical significance for all tests at $P < 0.05$.

Results

Myostatin and TLLs

Both GAPDH mRNA expression and protein levels did not differ significantly among groups, so GAPDH should serve and effective housekeeping role in this study. No significant differences were detected in pectoralis mRNA expression for myostatin or the TLLs among acclimation treatments (Figure 1). We detected a single band for the myostatin antibody in our Western blots, which correspond to the 52 kDa unprocessed latent form of myostatin. As for mRNA expression, protein expression of myostatin did not vary significantly among acclimation groups (Figure 2).

Trans-sarcolemmal and intramyocyte lipid transport

No significant differences among acclimation treatments were detected for pectoralis mRNA expression of *FAT/CD36* or *FABPpm* (Figure 1).

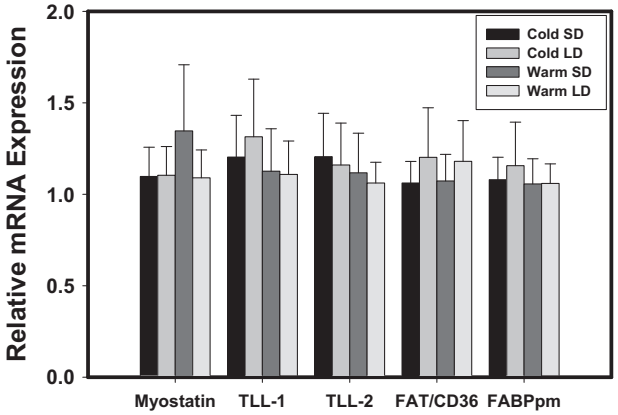

Figure 1. Temperature and photoperiod effects on relative mRNA expression levels from qRT-PCR for myostatin, tolloid-like protein 1 (TLL-1), tolloid-like protein 2 (TLL-2), plasma membrane-bound fatty acid binding protein (FABPpm), and fatty acyl translocase (FAT/CD36) in pectoralis muscles of dark-eyed junco (Junco hyemalis). Error bars represent SE. Sample sizes for the different treatment groups were: cold short day (Cold SD), $n = 11$; cold long day (Cold LD), $n = 10$; warm short day (warm SD), $n = 10$; warm long day (warm LD), $n = 10$.

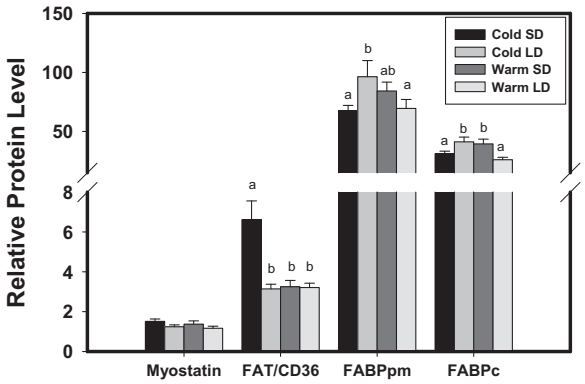

Figure 2. Temperature and photoperiod effects on relative protein levels from western blot for myostatin, tolloid-like protein 1 (TLL-1), tolloid-like protein 2 (TLL-2), plasma membrane-bound fatty acid binding protein (FABPpm), and fatty acyl translocase (FAT/CD36) in pectoralis muscles of dark-eyed junco (Junco hyemalis). Error bars represent SE. Sample sizes for the different treatment groups were: cold short day (Cold SD), $n = 11$; cold long day (Cold LD), $n = 10$; warm short day (warm SD), $n = 10$; warm long day (warm LD), $n = 10$. Different letters denote significant differences between treatment groups.

Table 2. Correlations among mass-independent metabolic rate residuals (from regressions of metabolic rate versus M_b

Pearson correlation coefficient

	M_{sum} residual	FAT/CD36 mRNA level	FABPpm mRNA level	FAT/CD36 Protein level	FABPpm Protein level	FABPc Protein level	CPT activity	CS activity	HOAD activity
M_{sum} Residuals		0.307	0.179	0.167	−0.092	0.127	0.278	0.144	0.185
mRNA expression									
FAT/CD36			0.783**	0.054	0.051	0.255	0.187	0.245	0.279
FABPpm				0.087	0.099	0.315*	0.116	0.338*	0.364*
Protein level									
FAT/CD36					−0.13	−0.026	0.108	−0.02	−0.172
FABPpm						0.763**	−0.103	0.253	0.196
$FABP_c$							−0.116	0.218	0.256
Enzyme activities									
CPT								0.345*	0.353*
CS									0.557**

Swanson et al. (2014b), activities of carnitine palmitoyl transferase (CPT), citrate synthase (CS), β-hydroxyacyl CoA-dehydrogenase (HOAD), protein levels of cytosolic fatty acid binding protein ($FABP_c$), and protein levels and mRNA expression of fatty acyl translocase (FAT/CD36) and plasma membrane-bound fatty acid binding protein (FABPpm) in pectoralis muscles of dark-eyed junco. $^*P < 0.05$; $^{**}P < 0.01$.

Pectoralis protein expression, however, showed more variation, with significant differences among acclimation treatments for all three fat transporters (Figure 2; FAT/CD36: $F_{1,40} = 11.734$, $P = 0.002$; FABPpm: $F_{1,40} = 6.053$, $P = 0.019$; $FABP_c$: $F_{1,40} = 13.741$, $P < 0.001$). FAT/CD36 protein expression was highest in the cold SD treatment and was significantly higher than for the other acclimation treatments. FABPpm protein expression was highest in the cold LD acclimation treatment and was significantly higher than cold SD and warm LD treatments. $FABP_c$ protein levels were significantly higher in the cold LD and warm SD than in the cold SD and warm LD groups.

Correlations

Neither pectoralis muscle mass residuals, myostatin mRNA or protein expression, fatty acid transporter mRNA or protein expression, nor cellular catabolic enzyme activities were significantly correlated with M_{sum} residuals (Tables 1 and 2). In the muscle remodeling pathway, mRNA expression of *TLL-1* and *TLL-2* were significantly positively correlated. In addition, myostatin protein expression was significantly positively correlated with *TLL-1* mRNA expression (Table 1). In the fatty acid transport and catabolism pathway, mRNA expression of *FAT/CD36* and *FABPpm* were strongly positively correlated. Protein expression of $FABP_c$ was also positively correlated with both mRNA and protein expression of FABPpm. In addition, *FABPpm* mRNA expression was significantly positively correlated with both CS and HOAD activities. Finally, CS, CPT, and HOAD enzyme activities were significantly positively correlated with each other (Table 2).

Discussion

Results from the present study suggest that regulation of lipid transporter protein levels in pectoralis muscle is complex, with variation in either temperatures or photoperiod potentially resulting in changes to lipid transport capacities. For example, high protein expression for all three lipid transporters occurred in cold temperatures, but high expression in the cold was dependent on photoperiod, with some transporter protein levels reaching maximum levels under cold and short days (FAT/CD36) and others under cold and long days (FABPpm and FABPc). Both of these responses could be interpreted as occurring to match to the higher energetic demands associated with cold and/or migratory status, the latter promoted by exposure of winter-collected birds to long days. Previous studies of these same juncos detected upregulation in genes and enzyme activities involved in fatty acid metabolism (Swanson et al. 2014b, Stager et al. 2015), but, importantly, this study extended these previous findings to protein expression in the pectoralis. Swanson et al. (2014c) previously documented for these same individual juncos that cold temperatures increased M_{sum}, but this was not associated with increased pectoralis muscle mass. In addition, CS and HOAD activities in these same juncos generally increased on long days, but activities were highest on the cold LD treatment and lowest on the warm SD treatment, potentially due to stimulation of the migratory disposition by long days (Swanson et al. 2014c). Stager et al. (2015) built upon these findings with analyses of genome-wide patterns of mRNA expression, documenting cold-induced increases in transcripts for muscle growth (although not *myostatin*), angiogenesis, and lipid transport and catabolic pathways.

We found little evidence in this study for upregulation of gene or protein expression of the myostatin system in pectoralis muscle in response to cold or short days. The absence of such an upregulation is consistent with the lack of an increase in pectoralis muscle mass in response to cold or short days (Swanson et al. 2014c) as well as the absence of a transcriptomic signature for the myostatin pathway (Stager et al. 2015) for these same juncos. Surprisingly, muscle remodeling mechanisms leading to hypertrophy, at least as mediated through the myostatin pathway, appear less important to temperature-induced variation in organismal metabolic capacities for dark-eyed juncos than is typical for the winter phenotype in birds (Swanson 2010). Collectively, these results suggests that enhanced lipid transport and catabolism capacities, rather than muscle hypertrophy, are one of the primary drivers of enhanced thermogenic capacity during cold acclimation for dark-eyed juncos.

Myostatin and TLLs

Myostatin, *TLL-1* and *TLL-2* mRNA expression and myostatin protein levels did not differ among acclimation treatments in this study. The qRT-PCR and Western blot analyses in the present study

validate the absence of variation in *myostatin* expression documented via transcriptomics in these same individual birds (Stager et al. 2015). Because pectoralis muscle mass also did not vary significantly among treatment groups for these birds (Swanson et al. 2014c), our results do not preclude a general role for the myostatin system in mediating seasonal muscle remodeling in birds. Even though increases in pectoralis muscle mass are a common component of phenotypic flexibility associated with winter or cold acclimation in many small birds (Vézina et al. 2006, 2007; Swanson 2010; Liknes and Swanson 2011b), a few previous studies have also documented an absence of pectoralis muscle mass changes for wintering (Swanson et al. 2014a) or migratory (King et al. 2015) phenotypes.

Trans-sarcolemmal lipid transport

Neither temperature nor photoperiod significantly altered pectoralis *FAT/CD36* and *FABPpm* mRNA expression in this study. Winter or migratory status in most bird species also fail to promote changes in mRNA expression for these two sarcolemmal lipid transporters (Zhang et al. 2015c, 2015d) and Stager et al. (2015) found no significant variation in mRNA expression of pectoralis *FAT/CD36* among temperature and photoperiod treatments for these same juncos. Moreover, cold and exercise training in house sparrows also did not change mRNA expression for *FAT/CD36* or *FABPpm* (Zhang et al. 2015a). In contrast, pectoralis muscle mRNA expression of FAT/CD36 was upregulated for captive white-throated sparrows photostimulated to migratory condition (Zajac et al. 2011) and yellow-rumped warblers *Setophaga coronata* showed higher *FAT/CD36* and *FABPpm* mRNA expression during spring migration than during fall migration (Zhang et al. 2015c). The present study detected a strong positive correlation in pectoralis mRNA, but not protein, expression between *FAT/CD36* and *FABPpm*, suggesting co-expression of these genes in response to acclimation treatments. Regarding the difference in mRNA and protein expression with acclimation, some studies in mammals have also failed to detect correlations between mRNA or protein expression and the rate of fatty acid transport into myocytes (Luiken et al. 2003; Chabowski et al. 2006). In contrast, some recent studies document that *FAT/CD36* and *FABPpm* are ubiquitously expressed (Nickerson et al. 2009), suggesting that the rate of trans-sarcolemmal fatty acid uptake depends on a cooperative role for the two fatty acid transporters (Chabowski et al. 2007). The positive correlation between pectoralis mRNA expression for *FAT/CD36* and *FABPpm* in this study provided support for a cooperative role between these two trans-sarcolemmal transporters in birds.

In contrast to results for mRNA expression in this study, temperature and photoperiod treatments in the present study both induced changes in pectoralis protein expression for both FAT/CD36 and FABPpm, with cold exposure effects being modified by photoperiod treatments. Pectoralis protein expression for these two transporters had different responses to photoperiod, with FAT/CD36 increasing on cold SD treatments and FABPpm increasing on cold LD treatments. Because protein levels of these sarcolemmal lipid transporters are more directly indicative of fatty acid transport capacities than mRNA expression, post-transcriptional processing of FABPpm and FAT/CD36 may alter protein levels (Bonen et al. 1999). As a consequence, fatty acid transporter protein expression might be expected to show more variation than mRNA expression (Glatz et al. 2010) with varying energy demands, as detected in the present study. Previous studies of wintering American goldfinches and cold-trained house sparrows both showed elevated pectoralis FAT/CD36 protein expression with incresing energy demands (Zhang et al. 2015a, 2015d). In addition, black-capped chickadees increased pectoralis FABPpm protein expression during winter compared to summer (Zhang et al. 2015d). Migratory birds may also increase pectoralis FABPpm protein levels during migration compared to non-migratory periods (McFarlan et al. 2009). Moreover, pectoralis FAT/CD36 protein expression increased during migration relative to summer for warbling vireos *Vireo gilvus* and yellow warblers *Setophaga petechia* and increased during spring relative to fall migration for yellow-rumped warblers (Zhang et al. 2015c). Pectoralis FABPpm protein levels also showed a similar seasonal pattern of variation for yellow and yellow-rumped warblers, but not for warbling vireos (Zhang et al. 2015c). Collectively, these studies suggest that pectoralis levels of the two sarcolemmal lipid transporters often increase in concert with increasing energy demands, but this is not always the case.

Reasons for the inconsistency between patterns of FAT/CD36 and FABPpm protein expression in the present study are unclear. Some studies have, however, documented different responses between these two fat transporters in skeletal muscles after contraction or insulin-treatment in mammals (Chabowski et al. 2004). These two proteins often act in conjunction at the plasma membrane, but translocation of FAT/CD36 and FABPpm to other locations in the myocyte under conditions of altered energy demand may differ (Chabowski et al. 2004; Han et al. 2007). Such differences in translocation could lead to different patterns of protein expression for the two sarcolemmal transporters, as documented in the present study.

Intramyocyte lipid transport

Variation in $FABP_c$ protein expression among acclimation treatments in this study was difficult to interpret, as levels increased on cold LD compared to cold SD and on warm SD compared to warm LD. Pectoralis intramyocyte lipid transport is consistently an important target of upregulation for migratory (Guglielmo et al. 2002; McFarlan et al. 2009) and winter (Liknes et al. 2014; Zhang et al. 2015d) phenotypes in birds. In addition, photo-stimulated migratory white-throated sparrows (Zajac et al. 2011) and cold- and exercise-trained house sparrows (Zhang et al. 2015a) also showed increases in $FABP_c$ expression. These data suggest that intracellular lipid transport is an important target of adjustment underlying elevation of metabolic capacities with increasing energy demands. The increase in $FABP_c$ on LD under cold treatment in this study supports the migratory disposition hypothesis, as exposure to a LD photoperiod for winter-collected birds could induce the spring migratory phenotype. On the other hand, within the warm treatment, SD birds had significantly higher $FABP_c$ levels than LD birds, possibly to maintain thermogenic function on a winter photoperiod. Complicating this interpretation further, Stager et al. (2015) documented cold-induced increases in pectoralis *$FABP_c$* mRNA expression for these same individual juncos. We were unable to amplify *$FABP_c$* mRNA in the present study, so we were unable to validate this finding.

Given the inconsistent results in the present study, it is, perhaps, important to note that $FABP_c$ serves not only as an intramyocyte fatty acid transporter, but also as a fatty acid receptor and may be co-regulated with fatty acid binding proteins on the cell membrane (Luiken et al. 2003). The membrane-bound and cytosolic forms of FABP in pectoralis were positively correlated in the present study. $FABP_c$ may also be more important to overall lipid transport capacity than membrane-associated lipid transporters because very

little fatty acid exists as free or unbound molecules inside muscle cells (Kiens 2006), whereas at least some fatty acid transport across membranes occurs by simple diffusion (Hamilton et al. 2002). Intramyocyte lipid transport capacity in birds may be especially important to organismal metabolic capacities because birds rely almost exclusively on exogenous lipids to fuel prolonged muscular activity (Jenni-Eiermann et al. 2002; Guglielmo 2010). In contrast, mammalian aerobic muscular activity is mainly fueled by carbohydrates and lipid droplets inside the myocyte (Guglielmo 2010). Despite the importance of FABPc to overall lipid transport in birds during elevated energy demands, protein expression was not consistently correlated with elevated energy demands for juncos in this study, suggesting complex interactions between temperature and photoperiod in the regulation of lipid transport in these birds.

Taken together, cold exposure, at least under some photoperiod conditions, increased all three fat transporters, suggesting that changes in this pathway could contribute to elevated M_{sum} under cold exposure in these same individual birds (Swanson et al. 2014c). However, photoperiod did not consistently alter either muscle mass, the myostatin system, or fatty acid transport pathways. This result is, perhaps, not surprising given that the transcriptomic study of Stager et al. (2015) on these same birds demonstrated increased concerted gene expression of the citric acid cycle and oxidative phosphorylation pathways on SD, but increased concerted gene expression in fatty acid metabolism pathways on LD. Because migratory phenotypes can be stimulated by either or both photoperiod or exercise (Price et al. 2011), it is possible that fuel transport and catabolism capacities did not change in the same direction due to photoperiod alone. Exercise might also be important for inducing changes in metabolic pathways and physiological responses for the migratory phenotype. Indeed, exercise training for house sparrows resulted in increases in pectoralis muscle mass, trans-sarcolemmal and intramyocyte lipid transport capacities and cellular metabolic intensities on constant photoperiods (Zhang et al. 2015a).

Correlations

In this study, neither pectoralis muscle mass, fat transport capacity, the myostatin system, nor regulatory enzymes for cellular metabolic intensity were significantly correlated with M_{sum}. Positive correlations of pectoralis muscle mass or cellular metabolic intensity with M_{sum} have been observed in previous avian studies (Petit and Vézina 2014; Swanson et al. 2013, 2014b). We observed significant positive correlations between protein expression of the active form of myostatin and mRNA expression of *TLL-1*, but not *TLL-2*, which suggests that TLL-1 serves as the major activator for myostatin in juncos. Prominent changes in mRNA expression of *TLL-1* but not *TLL-2* have also been observed for wintering house sparrows (Swanson et al. 2009), but this was not true for other bird species, as pectoralis *TLL-2* expression in winter black-capped chickadees exceeded that for summer but *TLL-1* expression did not, and similar seasonal trends for both TLLs occurred for American goldfinches (Swanson et al. 2014a). In addition, neither the migratory condition (King et al. 2015) nor exercise-training (Price et al. 2011) resulted in alterations of pectoralis mRNA expression for the TLLs relative to non-migratory or non-exercised conditions.

For fatty acid transporters, we observed a positive correlation of pectoralis *FAT/CD36* and *FABPpm* mRNA expression, emphasizing the importance of cooperation between these two sarcolemmal lipid transporters. Moreover, $FABP_c$ protein expression was positively correlated with both FABPpm mRNA and protein expression, highlighting the potential role for $FABP_c$ as a receptor for NEFAs from membrane-bound FABPpm. Similar correlations between these FABPs have also been observed for exercise- and acute cold-trained house sparrows (Zhang et al. 2015a). Together with the positive correlations of pectoralis CS with CPT and HOAD, the data suggest parallel variation and integration of the various steps in fatty acid transport and catabolism pathways in dark-eyed juncos in this study. Such relationships are consistent with the concept of symmorphosis and suggest that correlated variation is important for acclimatization to temperature and photoperiod in birds (Suarez, 1998, Swanson, 2010). Similar correlations among these pathways were also observed in exercise- and cold-trained house sparrows (Zhang et al. 2015a) and during migration in white-throated sparrows (McFarlan et al. 2009), but were not observed during migration or winter acclimatization for other birds (Zhang et al. 2015c, 2015d). As a result, evidence for the concept of symmorphosis of metabolic pathways under conditions of increased energy demands is still inconclusive, but is seemingly more consistent for experimental manipulation under standardized laboratory conditions than under natural conditions requiring elevated energy demands.

In conclusion, along with the data of Swanson et al. (2014c) and Stager et al. (2015), these data suggest that regulation of flight muscle mass, the myostatin system, and lipid transport capacities are common methods for regulating metabolic capacities of birds in general. Effects of cold acclimation or winter acclimatization, migratory status, or photoperiod on these pathways, however, might be species- or context-specific, so firm generalizations about regulation of these pathways under different conditions promoting increased energy demands are currently difficult to delineate.

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