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Phenotypic flexibility in passerine birds: Seasonal variation of aerobic enzyme activities in skeletal muscle

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ABSTRACT

Improved winter cold tolerance is widespread among small passerines resident in cold climates and is generally associated with elevated summit metabolic rate (M_{sum}=maximum thermoregulatory metabolic rate) and improved shivering endurance with increased reliance on lipids as fuel. Elevated M_{sum} and improved cold tolerance may result from greater metabolic intensity, due to mass-specific increase in oxidative enzyme capacity, or increase in the masses of thermogenic tissues. To examine the mechanisms underlying winter increases in M_{sum}, we investigated seasonal changes in mass-specific and total activities of the key aerobic enzymes citrate synthase (CS) and β-hydroxyacyl CoA-dehydrogenase (HOAD) in pectoralis, supracoracoideus and mixed leg muscles of three resident passerine species, black-capped chickadee (*Poecile atricapillus*), house sparrow (*Passer domesticus*), and white-breasted nuthatch (*Sitta carolinensis*). Activities of CS were generally higher in winter than in summer muscles for chickadees and house sparrows, but not nuthatches. Mass-specific HOAD activity was significantly elevated in winter relative to summer in all muscles for chickadees, but did not vary significantly with season for sparrows or nuthatches, except for sparrow leg muscle. These results suggest that modulation of substrate flux and cellular aerobic capacity in muscle contribute to seasonal metabolic flexibility in some species and tissues, but such changes play varying roles among small passerines resident in cold climates.

1. Introduction

Winter at temperate and arctic latitudes represents a period of energetic stringency for small birds because of cold ambient temperatures, decreased food abundance, reduced day length in which to forage, and associated long nights of forced fasting. Small birds resident in these climates show phenotypically flexible responses to winter cold resulting in a winter phenotype characterized by a marked improvement in cold tolerance (Dawson and Marsh, 1989; Marsh and Dawson, 1989a). Seasonal adjustments characterizing the winter phenotype may include elevated fattening (Dawson et al., 1983; Blem, 1990; Rogers and Smith, 1993), increased shivering endurance (Dawson and Marsh, 1989), increased thermogenic capacity, or summit metabolism (M_{sum}; reviewed in Swanson, 2010), changes in catabolic enzyme activity and substrate metabolism (reviewed in Marsh and Dawson, 1989b; Swanson, 2010), pectoralis muscle hypertrophy (Swanson, 1991; O'Connor, 1995a; Cooper, 2002; Swanson et al., 2009), and respiratory or circulatory changes resulting in increased oxygen delivery to muscles (Swanson, 1990; Arens and Cooper, 2005). Although winter increases in cold tolerance and M_{sum} are widespread among small birds in cold winter climates, the mechanistic underpinnings of these organismal adjustments are not always consistent among species and relatively few species have been studied. Therefore, a definitive mechanistic explanation for the marked winter improvement in cold tolerance in small birds remains elusive.

Winter increments of M_{sum} and cold tolerance are related to changes in skeletal muscle, which is the primary thermogenic organ in birds (Marsh and Dawson, 1989b). Elevated capacities for muscular thermogenesis in winter may involve changes in cellular aerobic capacity and/or skeletal muscle hypertrophy. Seasonal adjustment of cellular aerobic capacity potentially involves modulation of activities of key catabolic enzymes in oxidative pathways and/or activities of enzymes and transporters involved in substrate mobilization and delivery pathways (reviewed in Marsh and Dawson, 1989b; Swanson, 2010). To determine the role that biochemical adjustments in metabolic pathways and associated alterations of cellular aerobic capacity might play in seasonal acclimatization, we measured mass-specific and total (mass-specific activity × wet muscle mass) activities of citrate synthase (CS) and β-hydroxyacyl CoA-dehydrogenase (HOAD), key regulatory enzymes for aerobic metabolism (citric acid cycle

Abbreviations: CS, citrate synthase; HOAD, β-hydroxyacyl CoA-dehydrogenase; M_{sum}, summit metabolic rate; scc, supracoracoideus; pect, pectoralis

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and β-oxidation, respectively), in muscles of summer and winter acclimatized black-capped chickadees (*Poecile atricapillus*), house sparrows (*Passer domesticus*), and white-breasted nuthatches (*Sitta carolinensis*). These species all show large seasonal variations in M_{sum}, in excess of 25% (Hart, 1962; Cooper and Swanson, 1994; Liknes and Swanson, 1996; Arens and Cooper, 2005).

Evidence supporting a role for enzyme regulation in winter acclimatization of small passerine birds is, at present, equivocal. Some studies have shown elevated mass-specific activities of HOAD in winter suggesting increased flux of lipid metabolic substrates into the citric acid cycle (Marsh and Dawson, 1982; Yacoe and Dawson, 1983), while in other studies mass-specific activities of HOAD were seasonally constant or varied independently (Carey et al., 1989; O'Connor, 1995b). Similarly, mass-specific activities of HOAD are not universally elevated in migrants versus residents in a variety of birds (Marsh, 1981; Lundgren and Kiessling, 1985, 1986, 1988; Driedzic et al., 1993; Guglielmo et al., 2002). However, winter acclimatized and migratory birds generally have an increased capacity for lipid oxidation, whereas their capacity for oxidation of carbohydrates is less well correlated with variation in energy demand.

The importance of the capacity for fat catabolism in improved thermogenic capacity and cold tolerance in winter is uncertain because HOAD and oxidative capacity, as indicated by mass-specific activity of CS or cytochrome c oxidase (COX), do not necessarily increase concomitantly (Marsh and Dawson, 1989b). Increased HOAD activity may figure prominently in glycogen sparing, however (Marsh and Dawson, 1989b). Reciprocal regulation of lipid and carbohydrate metabolism resulting in preferential catabolism of non-esterified fatty acids (NEFA) and enhanced sparing of muscle glycogen in winter birds relative to summer birds has also been hypothesized to play a role in seasonal acclimatization (Marsh and Dawson, 1982; Marsh et al., 1990; Swanson, 1991), a hypothesis originally based on the correlation between glycogen depletion and fatigue in exercising mammals (Fitts et al., 1975; Gollnick, 1985).

In order for increased substrate flux to be effective as a mechanism of improving cellular aerobic capacity, enzyme activities of Krebs cycle and oxidative phosphorylation pathways, such as CS and COX, would need to be elevated as well. However, correlated changes in enzymes feeding substrates into the citric acid cycle and enzymes of the citric acid cycle or oxidative phosphorylation pathway are not always associated with changing energy demands in birds. Responses of CS or COX activities vary among different tissues and different species during seasonal acclimatization (Carey et al., 1989; O'Connor, 1995b; Liu et al., 2008; Zheng et al., 2008; 2010), migration (Marsh, 1981; Lundgren and Kiessling, 1985, 1986, 1988; Guglielmo et al., 2002), cold acclimation (Barré et al., 1987; Vittoria and Marsh, 1996), and exercise (Butler and Turner, 1988; Hammond et al., 2000). To summarize, while there is some evidence to support the hypothesis that highly aerobic activities, such as long-distance migration or prolonged shivering in cold climates, may stimulate an elevation in cellular aerobic capacity and substrate flux in birds, other evidence suggests that this is not a universal response. Our goal in this study was to investigate whether changes in cellular metabolic intensity in skeletal muscles contribute to the winter phenotype in three species of small, resident passerines that show marked seasonal changes in cold tolerance and M_{sum}.

2. Materials and methods

2.1. Birds

We captured wild, free-living adult birds by mist net before 12:30 CST in Union and Clay counties, South Dakota (approximately 42°40′N, 96°56′) in summer (mid-May to early September 1998–2002) and winter (December to early March 1998–2002). At capture, we weighed birds to the nearest 0.1 g using an Ohaus portable balance (Model LS200, Pine Brook, New Jersey). We determined age and gender in the field (Pyle, 1997), when possible, and confirmed initial classifications during dissection. We used approximately equal numbers of males and females in this study (male:female ratios = chickadees 32:29, sparrows 22:24, nuthatches 30:20). Following capture, we transported birds to the laboratory where they were caged at room temperature (20–25 °C) and held for 1–5 h until experiments began. However, because it was not always possible to run experiments on all the birds captured during their active phase, in a few instances birds were held out of doors overnight, and tested the next day. Animal handling and experimental protocols were approved by the University of South Dakota Institutional Animal Care and Use Committee and conformed to the guidelines of the Ornithological Council. We used only birds with completely ossified skulls (aged as adults) for enzyme measurements. We fed birds mealworms (*Tenebrio* sp.), sunflower seeds, and water *ad libitum* during holding periods prior to experiments. To determine whether cold stress modulated enzyme activities, we subjected birds to either the holding period only (controls) or to 1 h metabolic trials at either thermoneutral temperatures (30 ± 1 °C in air; thermoneutral controls) or severe cold exposure in helox (21% O_2, 79% He), at temperatures ranging from 6° to −3 °C (cold-exposed). Our cold exposure treatment involved subjecting individual birds to a single temperature within this range, with the temperature varying depending on season and species (i.e., colder temperatures for winter birds and for larger species). The cold exposure treatment was designed to provide a cold challenge producing metabolic rates approaching M_{sum}, but without birds becoming hypothermic before completion of the 1-h treatment period.

After metabolic trials or holding periods (for controls), we euthanized birds by cervical dislocation, then quickly excised muscles on ice. After we measured tissue wet masses to the nearest 0.01 g, tissues were flash-frozen in liquid nitrogen. The elapsed time between death and freezing of tissues to be assayed was less than 15 min, a time that O'Connor and Root (1993) found not to affect the activities of CS and HOAD in passerine skeletal muscle. We euthanized birds between 1200 and 1730 CST in winter and 1200 and 1900 CST in summer. We stored frozen tissues at −70 °C from 4 to 36 months.

To correct for any potential decay in activity with storage, we measured enzyme activities twice for some tissue samples. For these assays, we took subsamples from tissues immediately after removal of tissues from the ultracold freezer, during which time we kept tissues on ice, and after which we immediately returned tissues to the ultracold freezer for continued storage. We generated least-squares linear regressions of change in activity vs. time in storage for these tissue samples and then adjusted activities of all enzymes for time in storage using the regression equations, forced through the origin. Decay in activity was significant for CS ($F_{1,18} = 85.30$, $P < 0.001$, $R^2 = 0.83$), but not for HOAD ($F_{1,18} = 1.44$, $P = 0.24$, $R^2 = 0.07$).

2.2. Enzyme activities

We used citrate synthase (CS; E.C. 4.1.3.7) and β-hydroxyacyl CoA-dehydrogenase (HOAD; E.C. 1.1.1.35) as indicators of cellular aerobic capacity and fat oxidation capacity, respectively (Marsh, 1981; O'Connor, 1995b). After removal of pectoralis (pect), supracoracoideus (scc), and mixed leg muscles (*iliotibialis cranialis*, *iliotibialis lateralis*, *iliofibularis*, *femorotibialis externus*, *fibularis longus*, *tibialis cranialis*, *extensor digitorum longus*, *gastrocnemius complex*)

from storage at −70 °C, we minced samples, weighed them to the nearest 0.0001 g, and homogenized them in 10–40 volumes/mass of homogenizing buffer containing 100 mM phosphate and 2 mM EDTA at pH 7.3.

We performed spectrophotometric assays at 25 ± 2 °C using a Beckman DU 7400 spectrophotometer (Beckman Coulter, Inc., Fullerton, CA) at 412 nm (CS) or 340 nm (HOAD). For CS assays, we followed Srere (1969) and Bass et al. (1969), as modified by Marsh (1981). The CS assay medium contained 100 mM triethanolamine–HCl, 2.5 mM EDTA, 0.1 mM 5.5′-dithiobis-(2-nitrobenzoic acid), 0.2 mM acetyl-CoA, and 0.5 mM oxaloacetate at pH 7.5 in a final volume of 1.0 mL. For HOAD assays, we followed Bass et al. (1969), as modified by Marsh (1981). The HOAD assay medium contained 100 mM triethanolamine–HCl, 5 mM EDTA, 0.225 mM $NADH_2$, and 0.1 mM acetoacetyl-CoA at pH 7.0 in a final volume of 1.0 mL. We ran each sample in duplicate and used average values for subsequent calculations. We report mean mass-specific activities as μmol min^{-1} g fresh $mass^{-1}$ and mean total activities (mass-specific activity × wet muscle mass) as μmol min^{-1}. Seasonal variation in wet muscle masses for these same individuals is provided in Liknes and Swanson (2011).

2.3. Statistical analyses

We present data as means ± SE, unless otherwise specified. To compare group means, we used 2-way ANOVA with treatment and season as independent variables. Tukey's test was used for pairwise multiple comparisons. We tested for normality using Shapiro–Wilk's test and for homogeneity of variance using Levene's test prior to parametric analyses. In the event that assumptions of normality and/or homogeneity of variances were not met, we *ln* transformed data and repeated ANOVAs. In a few instances, *ln* transformation did not satisfy ANOVA assumptions, but because ANOVA is robust to small deviations from these assumptions, any error made would most likely be type-II and, therefore, conservative (Zar, 1996). If neither treatment nor the season∗treatment interaction were significant, we simplified the model by removing these terms and used a one-way ANOVA with season as the independent variable. We accepted $P < 0.05$ as a significant difference for all statistical comparisons. We performed all statistical tests using SAS statistical software (SAS Institute, 1990).

3. Results

Pectoralis CS activity was significantly greater in winter than in summer for chickadees on both mass-specific (μmol min^{-1} g FW^{-1}, 52%, $F_{1,29}=11.80$, $P=0.002$; Fig. 1) and total activity (μmol min^{-1}, 62.5%, $F_{1,29}=17.07$, $P<0.001$; Table 1) bases. Similarly, chickadee scc CS activity was significantly greater in winter than in summer for both mass-specific (38%, $F_{1,28}=13.95$, $P<0.001$; Fig. 1) and total activity (61%, $F_{1,28}=24.3$, $P<0.001$; Table 1) comparisons. Significant season∗treatment interactions occurred for chickadees for mass-specific ($F_{2,\ 23}=4.83$, $P=0.018$) and total activities ($F_{2,23}=6.26$, $P=0.007$) in scc, with higher activities during cold exposure in winter, but lower activities during cold exposure in summer. Mass-specific CS activity in sparrow pectoralis did not differ significantly between seasons (Fig. 1), but total activity was greater in winter than in summer (51.8%, $F_{1,26}=5.89$, $P=0.023$; Table 1). Sparrows did show significant winter increments of CS activities in scc for both mass-specific and total comparisons (mass-specific: 48.7%, $F_{1,26}=6.28$, $P=0.019$, Fig. 1; total: 59.3%, $F_{1,26}=7.19$, $P=0.013$; Table 1). Neither mass-specific nor total activities of CS varied significantly with season for nuthatches in either pect or scc (Fig. 1). Leg muscles showed significant winter increases in mass-specific CS activity only in chickadees, where winter values were greater than summer values for both mass-specific (42.7%, $F_{1,26}=8.16$, $P=0.008$; Fig. 1) and total (37.3%, $F_{1,26}=5.66$, $P=0.024$; Table 1) comparisons. No other treatment effects or season ∗ treatment interactions for CS activity were significant for any species or tissue.

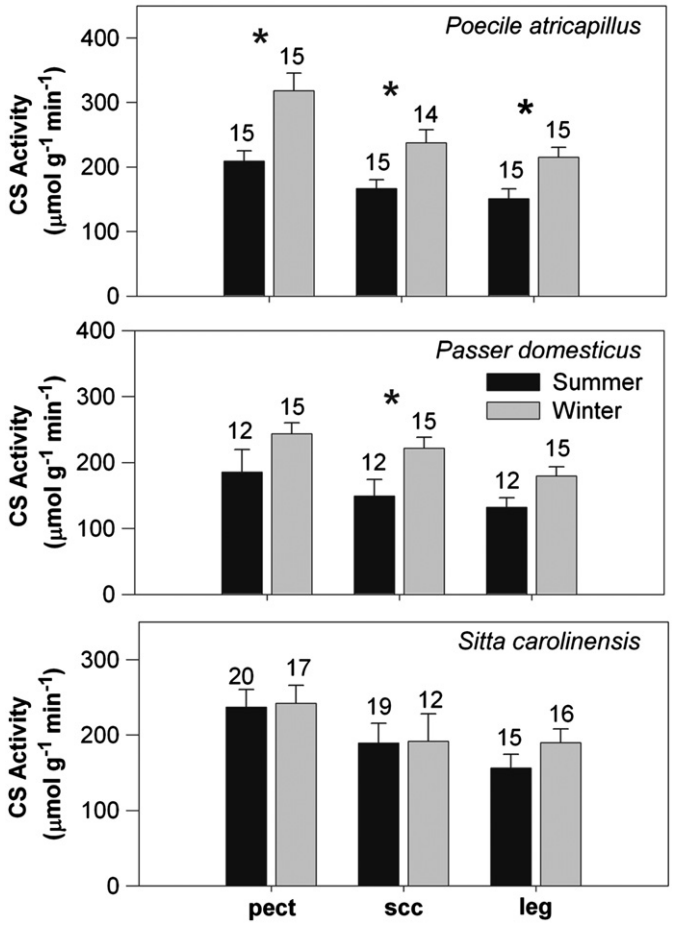

Fig. 1. Seasonal changes in citrate synthase (CS) mass-specific activity (μmol product g^{-1} min^{-1}), per gram of wet tissue. Solid bars represent summer activities and shaded bars represent winter activities for pectoralis (pect), supracoracoideus (scc), and mixed leg muscles for black-capped chickadees (*Poecile atricapillus*), house sparrows (*Passer domesticus*), and white-breasted nuthatches (*Sitta carolinensis*). Error bars represent SE. *Significant at $P \leq 0.05$.

Chickadees displayed a significant winter elevation in mass-specific HOAD activity for pect (88.3%, $F_{1,29}=29.78$, $P<0.001$, Fig. 2), scc (68.4%, $F_{1,29}=7.77$, $P=0.009$, Fig. 2) and leg muscle (102%, $F_{1,29}=11.83$, $P=0.002$, Fig. 2). Similarly, total HOAD activity was also significantly elevated in winter for all tissues for chickadees (pect: 117%, $F_{1,29}=48.19$, $P<0.001$; scc: 66.2%, $F_{1,29}=5.65$, $P=0.025$; leg: 95.3%, $F_{1,29}=5.82$, $P=0.023$; Table 1). Mass-specific seasonal HOAD activity did not vary significantly for sparrow pect or scc (Fig. 2). Similarly, total HOAD activity did not vary significantly between winter and summer for sparrow pect, but total HOAD activity was significantly greater in winter than in summer for sparrow scc (67.5%, $F_{1,26}=8.76$, $P=0.007$; Table 1). Sparrows also demonstrated significant winter increases in both mass-specific (43.8%, $F_{1,28}=4.99$, $P=0.034$; Fig. 2) and total (67.5%, $F_{1,28}=13.42$, $P=0.001$; Table 1) HOAD activity in leg muscle. HOAD activity did not vary significantly on a seasonal basis for any tissue, for either mass-specific or total comparisons,

Table 1
Mean (± SE) total enzyme activities (μmol min^{-1}) for citrate synthase (CS) and β-hydroxyacyl CoA-dehydrogenase (HOAD) in pectoralis, supracoracoideus and leg muscles during summer and winter for the three study species (cold and thermoneutral treatment groups pooled). Sample sizes are the same as in Figs. 1 and 2.

	CS		HOAD	
	Summer	**Winter**	**Summer**	**Winter**
Black-capped chickadee				
Pectoralis	374.0 ± 31.0	607.5 ± 47.2[c]	19.78 ± 2.71	37.69 ± 4.81[c]
Supracoracoideus	30.4 ± 3.1	50.2 ± 4.2[c]	5.56 ± 2.55	9.42 ± 3.32[a]
Leg	165.6 ± 16.1	227.3 ± 16.2[a]	10.86 ± 1.26	18.39 ± 3.21[a]
House sparrow				
Pectoralis	859.7 ± 163.3	1304.7 ± 99.5[a]	81.11 ± 14.81	90.53 ± 7.84
Supracoracoideus	72.1 ± 13.1	114.9 ± 9.6[a]	6.66 ± 0.93	11.18 ± 1.96[b]
Leg	269.3 ± 54.7	363.0 ± 33.9	21.90 ± 3.48	35.84 ± 4.24[c]
White-breasted nuthatch				
Pectoralis	479.8 ± 70.1	829.4 ± 76.9	44.15 ± 4.83	47.17 ± 3.67
Supracoracoideus	28.6 ± 8.3	56.7 ± 10.8	6.47 ± 2.34	3.65 ± 0.25
Leg	267.0 ± 30.9	310.8 ± 29.1	16.59 ± 2.37	19.76 ± 1.27

[a] $P < 0.05$.
[b] $P < 0.01$.
[c] $P < 0.001$.

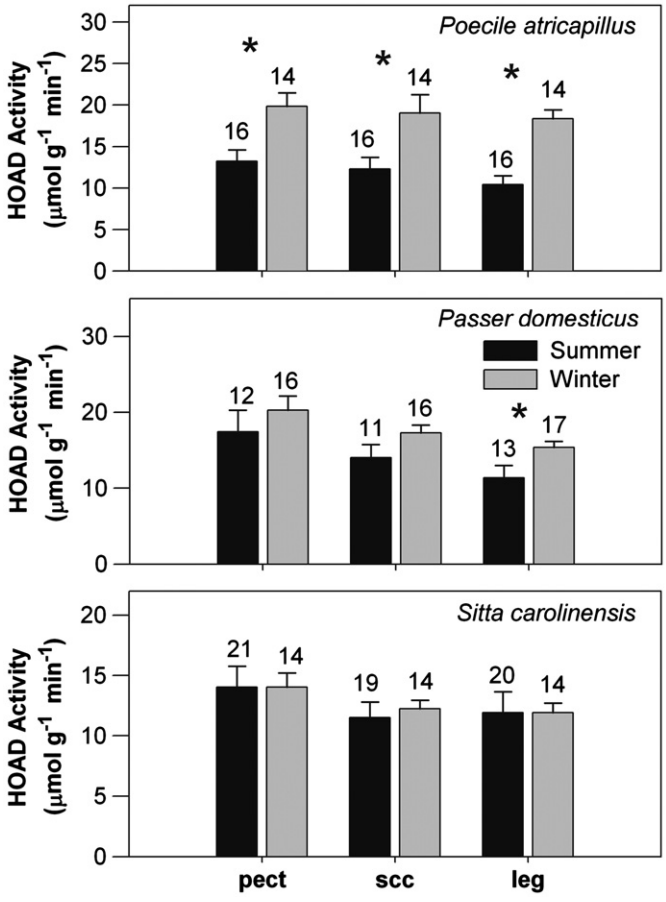

Fig. 2. Seasonal changes in β-hydroxyacyl CoA-dehydrogenase (HOAD) mass-specific activity (μmol product g^{-1} min^{-1}), per gram of wet tissue. Solid bars represent summer activities and shaded bars represent winter activities for pectoralis (pect), supracoracoideus (scc), and mixed leg muscles for black-capped chickadees (*Poecile atricapillus*), house sparrows (*Passer domesticus*), and white-breasted nuthatches (*Sitta carolinensis*). Error bars represent SE. *Significant at $P \leq 0.05$.

in nuthatches (Fig. 2, Table 1). No treatment effects or interaction terms were significant for any species or any tissue for HOAD activity.

4. Discussion

4.1. Comparisons among species

Precise comparisons of enzyme activities among studies are difficult because differences in protocols, assay temperatures and equipment vary among studies and may affect enzyme activities. Nevertheless, such comparisons can validate results, suggest general trends among species and treatments and stimulate questions for additional research. Pectoralis CS activities for the three species in our study ranged from 185 to 318 μmol g^{-1} min^{-1} (Fig. 1) and are consistent with those for other small birds, which range from 100 to 375 μmol g^{-1} min^{-1} (Marsh, 1981; Marsh and Dawson, 1982; Yacoe and Dawson, 1983; Lundgren and Kiessling, 1985, 1986, 1988; Carey et al., 1989; Driedzic et al., 1993; O'Connor, 1995b; Guglielmo et al., 2002; Dawson and Olson, 2003). The highest recorded CS activity for avian flight muscle (combined pectoralis and supracoracoideus) is from rufous hummingbirds (*Selasphorus rufus*) at 448 μmol g^{-1} min^{-1} (Suarez et al., 1990).

Fewer studies have examined CS activities in supracoracoideus and leg muscles in birds. Mean supracoracoideus CS activity was 90.3 μmol g^{-1} min^{-1} for gray catbirds (Marsh, 1981), a value slightly lower than our values, which range from 149 to 237 μmol g^{-1} min^{-1} (Fig. 1). Our values for leg muscle CS activity range from 132 to 215 μmol g^{-1} min^{-1} and are somewhat higher than leg muscle CS activities for other small birds, which range from 19 to 80 μmol g^{-1} min^{-1} (Marsh and Dawson, 1982; Carey et al., 1989; Choi et al., 1993; Dawson and Olson, 2003), perhaps suggesting that leg muscles are important for thermogenesis in our study species, as no obvious differences in the use of legs for perching or locomotion occur among our study species and those in other studies.

HOAD activities in pectoralis muscles from small birds range from 11 to 92 μmol g^{-1} min^{-1} (Marsh, 1981; Marsh and Dawson, 1982; Yacoe and Dawson, 1983; Lundgren and Kiessling, 1985, 1986, 1988; Carey et al., 1989; O'Connor, 1995b; Guglielmo et al., 2002; Dawson and Olson, 2003), and activities for the three species in our study (13–20 μmol g^{-1} min^{-1}, Fig. 2) are at the lower end of this range. Similarly, HOAD activities in supracoracoideus and leg muscles in our study species are consistent with those for other small birds. Supracoracoideus HOAD activity averaged 21.1 μmol g^{-1} min^{-1} in gray catbirds (Marsh, 1981) and 11–19 μmol g^{-1} min^{-1} for our study species (Fig. 2). Leg muscle HOAD activities for small birds range

from 6 to 18 μmol g^{-1} min^{-1} (Marsh and Dawson, 1982; Carey et al., 1989; Dawson and Olson, 2003), similar to leg muscle HOAD activities in our study species (10–18 μmol g^{-1} min^{-1}, Fig. 2). These data suggest roughly similar muscular capacities for lipid oxidation in small birds generally.

4.2. Seasonal comparisons

CS activity was elevated in winter relative to summer for all muscles for chickadees and sparrows, significantly so for all muscles except sparrow pectoralis and leg. To our knowledge, the winter increases in flight muscle CS activity for chickadees and sparrows are the first documented occurrences of such increases as components of winter acclimatization in birds, although flight muscle cytochrome c oxidase activity, another indicator of cellular aerobic capacity, may increase in winter (Liu et al., 2008; Zheng et al., 2008, 2010). In contrast, nuthatches did not exhibit elevated winter CS activity in any muscle and seasonal values were very similar. Although seasonal variation of flight muscle CS has not been previously documented in winter acclimatized passerines, studies have been limited to Cardueline finches (Marsh and Dawson, 1982; Yacoe and Dawson, 1983; Carey et al., 1989; O'Connor, 1995b). Increments of mass-specific aerobic capacity are correlated with migratory status in some birds (Lundgren and Kiessling, 1985, 1986; Guglielmo et al., 2002), but not in others (Marsh, 1981; Lundgren and Kiessling, 1985; Driedzic et al., 1993; Weber and Piersma, 1996). Interestingly, while mass-specific pectoralis activity of CS did not vary significantly between summer and winter American goldfinches (*Carduelis tristis*), CS activity in spring was significantly greater than in summer (Yacoe and Dawson, 1983). Similar to our data for chickadees, significant winter elevation in mass-specific CS activity has been documented in leg muscles of house finches (*Carpodacus mexicanus*; Carey et al., 1989), but such variation was not present in sparrows or nuthatches in our study. Thus, although variation in muscle CS activity and cellular aerobic capacity may contribute to seasonal acclimatization or migratory disposition in some birds, it is not a universal adjustment promoting elevated aerobic capacity under conditions of high aerobic energy demand.

Maximum metabolic rates during flight or exercise are higher than those during thermogenesis in birds (Marsh and Dawson, 1989b; Wiersma et al., 2007; Swanson, 2010), so the possibility exists that the aerobic capacity of flight muscles provides sufficient capacity for thermogenesis at all seasons, without specific adjustment to cold temperatures. Such a possibility is consistent with the absence of seasonal variation in mass-specific CS activity in pectoralis of several species of small birds (Marsh and Dawson, 1982; Yacoe and Dawson, 1983; Carey et al., 1989; O'Connor, 1995b; this study). However, cellular aerobic capacity of pectoralis muscle does increase in winter for several species of small passerine birds (Liu et al., 2008; Zheng et al., 2008, 2010), including the black-capped chickadee in this study. Moreover, winter increases in M_{sum} and flight muscle mass relative to summer are a consistent component of the winter phenotype in small birds (Swanson, 2010), including the species in this study (Liknes and Swanson, 2011), suggesting that winter acclimatization does promote increases in some aspects of muscular aerobic capacity. These data suggest the possibility that aerobic capacity for exercise and thermogenesis may not be tightly correlated. Indeed, maximum metabolic rate (MMR) during hop-flutter wheel exercise was not correlated with M_{sum} in tropical birds (Wiersma et al., 2007). In contrast, adaptation for migratory condition in red knots (*Calidris canutus*) does confer thermogenic side effects, so adjustment of aerobic capacity for migratory flight may increase both MMR and M_{sum} in some birds. Thus, the relationship between flight and thermogenic metabolic rates in birds and the degree of overlap between physiological and biochemical mechanisms promoting elevated metabolic rates remain to be fully characterized.

One additional factor may relate to variable seasonal responses to cellular aerobic capacity among species, namely body size. The black-capped chickadee demonstrated the greatest winter increment of mass-specific pectoralis CS activity in this study and was the only species to show consistent significant winter increases (38–52%) of mass-specific CS activity in all muscles. Chickadees were also the smallest of the three species in this study (12–14 g vs. 26–29 g for house sparrows and 19–21 g for white-breasted nuthatches; Liknes and Swanson, 2011), so perhaps small body size, and the accompanying higher surface area to volume ratio, may exacerbate heat loss in cold temperatures and elicit greater seasonal adjustments in cellular aerobic capacity in smaller bird species. Documentation of whether such a relationship between body size and the magnitude of phenotypic flexibility of aerobic capacity occurs generally in birds will require further research.

Muscle CS activity appears to be very flexible in mammals, being influenced, for example, by acclimation/acclimatization (Wickler, 1981; Walters and Constable, 1993), training (Starritt et al., 1999; Siu et al., 2003), and acute exercise (Leek et al., 2001). Measured CS activity is often highly variable, even when similar protocols are employed and samples are taken from the same tissues (Leek et al., 2001). The same may be true for birds, although the absence of significant treatment effects for any enzyme in any tissue for any of our study species suggests that acute cold exposure and the accompanying elevation of shivering thermogenesis do not impact aerobic enzyme activities in small birds. Nevertheless, the significant season by treatment interaction for supracoracoideus CS activity in chickadees suggests the possibility of some flexibility in CS function, perhaps through regulation of allosteric modifiers, in response to acute cold exposure. If CS activity is flexible in birds, variation in CS activity in response to different levels of aerobic conditioning may be missed because of experimental designs that fail to control for short-term variation (on the order of days to weeks) in factors influencing aerobic capacity. Indeed, Vézina and Williams (2005) found significant variation in CS activity for European starlings (*Sturnus vulgaris*) in different breeding stages within the breeding season. If CS activity is flexible over periods of days to weeks in birds, studies grouping birds into coarse categories such as season or migratory status might result in high variance in CS activity, which could obfuscate finer-scale CS variation within seasons or among different migratory stages, where organismal metabolic variation has been documented (Piersma et al., 1999; Swanson and Olmstead, 1999).

HOAD activity was significantly elevated in winter for all muscles in chickadees, but the only significant seasonal difference for the other two species was a winter increase for sparrow leg muscle. Elevated mass-specific pectoralis activities of HOAD have been reported in winter acclimatized American goldfinches (Marsh and Dawson, 1982; Yacoe and Dawson, 1983) and house finches from Colorado (Carey et al., 1989), but not from Michigan house finches (O'Connor, 1995b). Colorado house finches also displayed a mass-specific winter increase in HOAD activity in leg muscle (Carey et al., 1989), but American goldfinches did not (Marsh and Dawson, 1982). Increased HOAD is often associated with improved endurance and, to a lesser extent, aerobic capacity in birds (Marsh and Dawson, 1989a; Swanson, 2010), but our data do not support the conclusion that HOAD activity in muscles is generally associated with increased thermogenic demand in small birds. None of the three species in our study show large seasonal changes in depot fat and plasma metabolite levels do not suggest an increased reliance on adipose-tissue derived (i.e., exogenous)

lipids as fuel for shivering muscles in winter (Liknes, 2005). However, chickadees and nuthatches show elevated winter levels of pectoralis fatty acid binding protein (an intracellular lipid transporter), which suggests an improved capacity for intracellular lipid transport, consistent with a greater reliance on exogenous lipid as a winter fuel source (Liknes, 2005). The elevated HOAD activity in winter relative to summer chickadees is consistent with an increased reliance on lipids to fuel shivering. Nuthatches and sparrows, however, showed little seasonal variation in HOAD activity, suggesting that cellular β-oxidative capacity in summer is also sufficient to meet winter demands in these species, at least for flight muscles, which are the major thermogenic organs in birds (Marsh and Dawson, 1989b; Dawson and Olson, 2003).

In conclusion, elevations of muscle CS and HOAD activity apparently serve a role in winter acclimatization and seasonal metabolic flexibility in some, but not all, small birds wintering in cold climates. These data suggest that cellular mechanisms promoting seasonal metabolic flexibility may vary among species, even when they share the same general pattern of acclimatization at the organismal level (i.e., large seasonal changes in organismal metabolic rates), and such changes do not appear to be universal components of seasonal adjustments to variable energy demands in small birds.

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