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RESEARCH ARTICLE

Relative roles of temperature and photoperiod as drivers of metabolic flexibility in dark-eyed juncos

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ABSTRACT

Seasonal phenotypic flexibility in small birds produces a winter phenotype with elevated maximum cold-induced metabolic rates (=summit metabolism, $\dot{M}_{sum}$). Temperature and photoperiod are candidates for drivers of seasonal phenotypes, but their relative impacts on metabolic variation are unknown. We examined photoperiod and temperature effects on $\dot{M}_{sum}$, muscle masses and activities of key catabolic enzymes in winter dark-eyed juncos (*Junco hyemalis*). We randomly assigned birds to four treatment groups varying in temperature (cold=3°C; warm=24°C) and photoperiod [short day (SD)=8 h:16 h light:dark; long day (LD)=16 h:8 h light:dark] in a two-by-two design. We measured body mass (M_b), flight muscle width and $\dot{M}_{sum}$ before and after 3 and 6 weeks of acclimation, and flight muscle and heart masses after 6 weeks. $\dot{M}_{sum}$ increased for cold-exposed, but not for warm-exposed, birds. LD birds gained more M_b than SD birds, irrespective of temperature. Flight muscle size and mass did not differ significantly among groups, but heart mass was larger in cold-exposed birds. Citrate synthase, carnitine palmitoyl transferase and β-hydroxyacyl Co-A dehydrogenase activities in the pectoralis were generally higher for LD and cold groups. The cold-induced changes in $\dot{M}_{sum}$ and heart mass parallel winter changes for small birds, but the larger M_b and higher catabolic enzyme activities in LD birds suggest photoperiod-induced changes associated with migratory disposition. Temperature appears to be a primary driver of flexibility in $\dot{M}_{sum}$ in juncos, but photoperiod-induced changes in M_b and catabolic enzyme activities, likely associated with migratory disposition, interact with temperature to contribute to seasonal phenotypes.

KEY WORDS: Phenotypic flexibility, Summit metabolic rate, Photoperiod, Temperature, Dark-eyed juncos, Birds, Pectoralis, Citrate synthase, β-hydroxyacyl-CoA-dehydrogenase, Carnitine acyl transferase

INTRODUCTION

Reversible phenotypic flexibility allows individual organisms to better match phenotypes to environmental or ecological demands, and such flexibility can have adaptive consequences (Piersma and Drent, 2003; Nussey et al., 2005; Piersma and van Gils, 2011). Further understanding of the adaptive consequences of phenotypic flexibility requires detailing not only patterns of phenotypic flexibility (e.g. Broggi et al., 2005; McKechnie, 2008), but also mechanisms underlying reversible phenotypic variation and the time scales over which such variation occurs (Piersma and Drent, 2003).

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One well-known example of reversible phenotypic flexibility is the seasonal phenotypes occurring in small birds in response to seasonal climatic variation (Swanson, 2010). The winter phenotype in these birds is characterized by improved cold tolerance and enhanced capacities for thermogenesis, and winter increases in summit metabolic rates ($\dot{M}_{sum}$; maximal thermoregulatory metabolic rates) of 10–50% are common among small birds (Swanson, 2010). Migratory birds also often show $\dot{M}_{sum}$ increases of a similar magnitude as a component of the migratory phenotype (Swanson, 1995; Swanson and Dean, 1999; Vézina et al., 2006; Vézina et al., 2007). Such increments of $\dot{M}_{sum}$ are associated with improved shivering endurance and heightened levels of cold tolerance, suggesting that physiological and biochemical changes responsible for altering $\dot{M}_{sum}$ also affect cold tolerance (Marsh and Dawson, 1989; Swanson, 2001; Swanson and Liknes, 2006). Moreover, high $\dot{M}_{sum}$ has positive fitness consequences for endotherms wintering in cold climates (Hayes and O'Connor, 1999; Sears et al., 2006; Clavijo-Baquet and Bozinovic, 2012) and presumably is related to higher endurance capacity for flight in migratory birds (Swanson and Dean, 1999; Vézina et al., 2007).

Because regulatory thermogenesis is primarily accomplished by muscular shivering in small birds, phenotypic flexibility should be most evident in skeletal muscle, especially flight muscles, which are the principal thermogenic organ (Hohtola, 1982; Hohtola et al., 1998; Dawson and O'Connor, 1996). At the level of skeletal muscle, increments of thermogenic capacity could be mediated by enhanced cellular metabolic intensity and/or by increases in muscle mass. Several studies have examined the influence of organ mass variation on individual variation in summit metabolism or maximum metabolic rates during exercise, and these studies suggest that positive correlations between pectoralis mass and $\dot{M}_{sum}$ or maximum exercise metabolic rate are common and that flight muscle masses can have an important influence on organismal aerobic capacity in birds (Chappell et al., 1999; Hammond et al., 2000; Vézina et al., 2006; Vézina et al., 2007; Swanson et al., 2013). Indeed, seasonal metabolic flexibility in small birds is generally associated with pectoralis muscle hypertrophy in winter or migratory condition, and percent seasonal changes in pectoralis muscle mass often rather closely parallel percent changes in $\dot{M}_{sum}$ (Swanson, 2010; Liknes and Swanson, 2011a). Modification of muscle size is thus a common mechanism by which birds mediate metabolic responses to changing energy demands (Piersma and van Gils, 2011).

Seasonal modulation of cellular metabolic intensity in flight muscles may also contribute to winter or migratory phenotypes in small birds (Marsh and Dawson, 1982; Swanson, 2010; Liknes and Swanson, 2011b). Such variation in cellular metabolic intensity is often measured by examining variation in activities of citrate synthase (CS; a key regulatory enzyme of the Krebs cycle) or cytochrome *c* oxidase (COX; a key regulatory enzyme of oxidative phosphorylation). Numerous examples of winter or migratory increases in CS or COX activities exist, but such changes do not

The Journal of Experimental Biology

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List of symbols and abbreviations

CPT	carnitine palmitoyl transferase
CS	citrate synthase
HOAD	β-hydroxyacyl Co-A dehydrogenase
LD	long day
M_b	body mass
$\dot{M}_{sum}$	summit metabolic rate (maximum thermogenic metabolic rate)
SD	short day
S/P ratio	ratio of supracoracoideus mass to pectoralis mass

occur universally among small birds (Dawson et al., 1983; Zheng et al., 2008; Swanson, 2010; Liknes and Swanson, 2011b). This suggests that seasonal variation in muscle mass is perhaps generally more important as a driver for metabolic flexibility in small birds than is variation in cellular metabolic intensity (Swanson et al., 2013), but nevertheless seasonal changes in cellular metabolic intensity do contribute to seasonal metabolic flexibility in some species.

Seasonal increases in shivering capacity promoted by pectoralis hypertrophy or cellular metabolic intensity necessitate increased fuel delivery to shivering muscles, and prolonged shivering and exercise in birds are fueled largely by lipid (Dawson et al., 1983; Vaillancourt et al., 2005; Vaillancourt and Weber, 2007). Moreover, enhanced fat catabolism capacity is a consistent component of winter and migratory phenotypes in small birds (Dawson et al., 1983; McWilliams et al., 2004; Swanson, 2010), which suggests that fat catabolism capacity may contribute directly to enhanced thermogenic or exercise performance. Such increases in fat catabolism capacity could involve adjustments to fat mobilization, transport or oxidation, but seasonal changes in this pathway have not been fully explored for small birds. Enhanced mass-specific fat oxidation capacity in pectoralis muscles, as measured by the activity of β-hydroxyacyl Co-A dehydrogenase (HOAD), occurs with winter acclimatization or migratory condition in some species, but such increases are not obligatory components of winter or migratory phenotypes (Dawson et al., 1983; McWilliams et al., 2004; Swanson, 2010; Guglielmo, 2010). This suggests that muscle lipid oxidation capacity is not strictly limiting to fat catabolic capacity in small birds.

Capacities for delivery of exogenous lipid to muscle cells are greater in birds than in mammals, and birds rely on exogenous lipid stores to fuel activity metabolism to a greater extent than mammals (McWilliams et al., 2004; Guglielmo, 2010). Transport capacities of lipids from blood plasma to mitochondria in skeletal muscle cells are possible candidates for mediation of seasonal variation in lipid catabolism capacities. Such transport involves several steps, including transfer across the sarcolemma, intracellular transport to the mitochondria, and transport across the mitochondrial membranes to the matrix where β-oxidation occurs (McWilliams et al., 2004; Guglielmo, 2010). Transport of free fatty acids into the mitochondrial matrix is catalyzed by carnitine acyl transferase (CAT) and CAT activity is upregulated during migration in birds (Driedzic et al., 1993; Guglielmo et al., 2002). These findings suggest that the capacity for free fatty acid transport into the mitochondrion is enhanced during migration, but whether this result applies to birds wintering in cold climates is unknown. Price et al. (Price et al., 2011) found that CAT activity varied with the fatty acid serving as substrate, with higher activities associated with increasing degree of unsaturation and shorter chain length. Such changes can affect metabolic performance in birds, as depot fat with higher levels of unsaturated fatty acids produces elevated maximal metabolic output (Pierce et al., 2005). Thus, the transport of fats across mitochondrial membranes seems a likely target for seasonal modulation of fat catabolic capacity in small birds.

Because parallel seasonal changes in flight muscle size, cellular metabolic intensity and $\dot{M}_{sum}$ are common in small birds (Swanson, 2010; Swanson and Merkord, 2013), this suggests that such variation may be mediated by predictable environmental cues such as photoperiod, and changes in photoperiod can induce changes in organismal metabolic rates in birds (Carey and Dawson, 1999). However, organismal metabolic rates may also vary among and within winters, and short- and medium-term temperature variables (0–30 days) were better predictors of $\dot{M}_{sum}$ and basal metabolic rate for small birds than long-term average temperatures (Swanson and Olmstead, 1999). This suggests that proximate temperature factors can also play an important role in mediating phenotypic flexibility in small birds. Numerous examples of metabolic variation in response to temperature acclimation exist for birds (McKechnie, 2008; McKechnie and Swanson, 2010). However, the relative roles of ultimate and proximate factors in the regulation of seasonal phenotypic flexibility in birds are unknown and experiments addressing relative roles of photoperiod and temperature as cues for seasonal phenotypic flexibility have been suggested as important means of understanding drivers of such flexibility (Carey and Dawson, 1999; Swanson, 2010).

In this study, we acclimated captive dark-eyed juncos (*Junco hyemalis* Linneaus), a common winter resident in South Dakota showing seasonal variation in pectoralis mass and $\dot{M}_{sum}$ (Swanson, 1990; Swanson, 1991a), to varying photoperiod [long-day (LD) and short-day (SD)] and temperature (cold and warm) treatments and examined the relative impacts of these treatments on phenotypic flexibility of body mass (M_b), $\dot{M}_{sum}$, muscle and heart masses, and activities of key aerobic enzymes important to cellular metabolic intensity. We hypothesize that cold exposure is most important as a direct effector of metabolic flexibility in juncos, so we predict that $\dot{M}_{sum}$, flight muscle masses and potentially cellular metabolic intensity will be higher in cold-acclimated than warm-acclimated birds. Photoperiod may also indirectly induce summer to winter metabolic variation, and if so, then trait values should be higher in SD birds than in LD birds for any given temperature, and cold SD birds should show the highest trait values and warm LD birds the lowest trait values. However, induction of migratory disposition in winter juncos by LD photoperiods has long been known (Wolfson, 1952; Eyster, 1954; Farner et al., 1961; Johnston, 1962), and migratory condition often increases $\dot{M}_{sum}$, flight muscle masses and cellular metabolic intensity in birds (Swanson, 2010; Piersma and van Gils, 2011). Thus, if exposure to LD induces migratory disposition, an alternative hypothesis is that LD birds may show higher trait values than SD birds within temperature treatments, with cold LD birds having the highest and warm SD birds the lowest trait values.

RESULTS

Body mass and $\dot{M}_{sum}$

M_b did not differ significantly among groups at the beginning of the temperature–photoperiod treatments (Fig. 1), and overall mean M_b was 17.9±0.1 g (n=44). The best-supported model for M_b included fixed effects for time, photoperiod and an interaction between the two, and a random effect of individual. Significant fixed-effects terms in the model were time ($F_{2,81}$=71.42, P<0.001), with M_b increasing with acclimation duration, and the photoperiod × time interaction ($F_{2,81}$=34.16, P<0.001), with M_b increasing with time to a greater extent for LD treatment groups than for SD treatment groups (Fig. 1). Photoperiod did not significantly influence M_b

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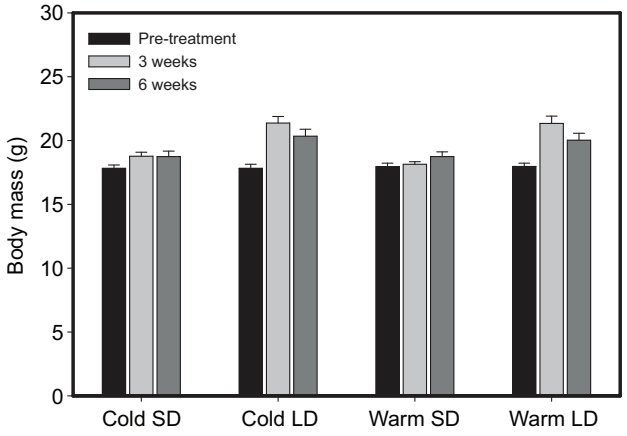

Fig. 1. Variation in mean ± s.e.m. body mass (M_b) among temperature–photoperiod acclimation groups for dark-eyed juncos. Mixed model analyses included time and the photoperiod × time interaction as significant predictors of M_b, with M_b increasing with time to a greater extent for long-day (LD) than for short-day (SD) treatment groups.

independent of time. The best-supported model for M_b did not include temperature or the interactions temperature × time, temperature × photoperiod or temperature × photoperiod × time.

At the initiation of the temperature–photoperiod treatments, $\dot{M}_{sum}$ did not differ significantly among treatment groups and mean $\dot{M}_{sum}$ was 6.005±0.106 ml O_2 min^{-1} (n=44). The best-supported model for $\dot{M}_{sum}$ included fixed effects for M_b (at time 0), temperature, time and a temperature × time interaction, and allowed for differences in the spread of residuals for the three time periods. As expected, $\dot{M}_{sum}$ increased significantly with increasing M_b ($F_{1,122}$=25.17, P<0.001; Fig. 2). All of the fixed effects in the model exerted a significant influence on $\dot{M}_{sum}$, including time ($F_{2,121}$=7.22, P=0.001), temperature ($F_{1,122}$=7.40, P=0.008) and the time × temperature interaction ($F_{2,121}$=15.90, P<0.001). $\dot{M}_{sum}$ was higher at cold than at warm temperatures (Fig. 2), and increased with time to a greater extent for cold than for warm treatment groups (Fig. 2B). Neither photoperiod nor the interactions between photoperiod × time, photoperiod × temperature or photoperiod × temperature × time were included in the best-supported model.

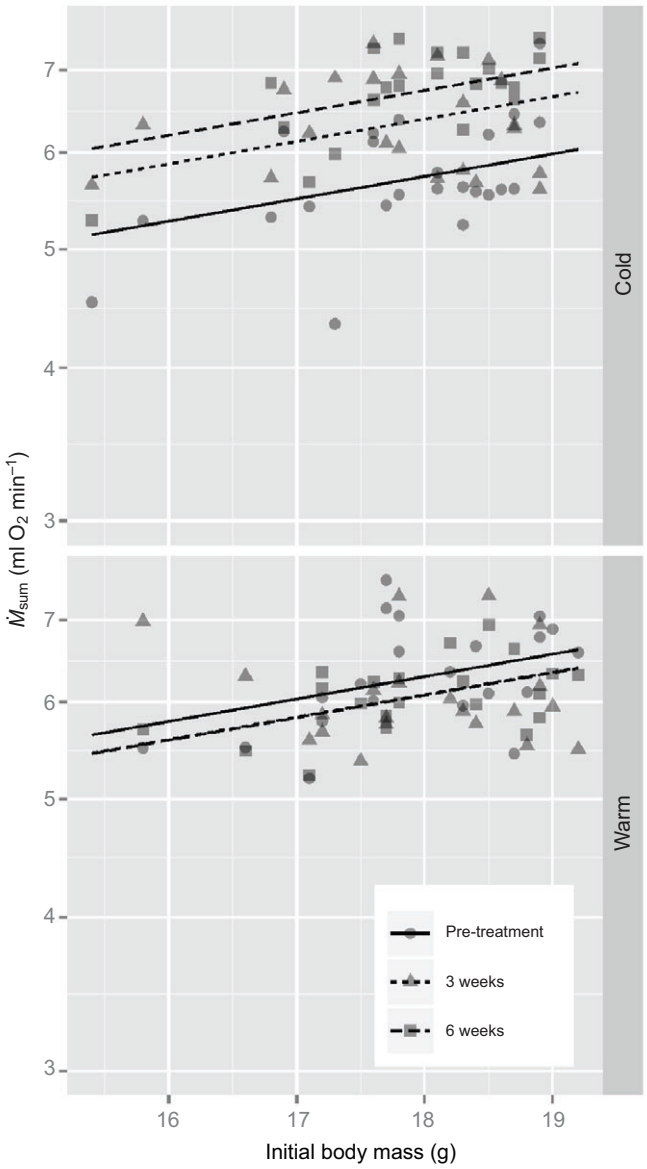

Fig. 2. Summit metabolic rate ($\dot{M}_{sum}$) as a function of initial body mass (body mass at time 0) at 0, 3 and 6 weeks of acclimation for cold and warm acclimation groups for dark-eyed juncos. $\dot{M}_{sum}$ increased significantly over time for cold-acclimated juncos but not for warm-acclimated juncos. An effect of photoperiod was not included in the best-supported model.

Ultrasound and muscle masses

Ultrasound measurements of flight muscle width were significantly positively related to flight muscle mass (combined pectoralis and supracoracoideus masses, one side only) according to the following equation: flight muscle mass (g)=0.904+[0.151×muscle width (mm)] (n=41, R^2=0.209, P<0.001).

Repeatabilities, calculated as the intraclass correlation coefficient (Lessells and Boag, 1987) for flight muscle width within each time period (0, 3 and 6 weeks) were 0.881, 0.865 and 0.870, respectively. Terms included in the best-supported model for ultrasound muscle width were M_b (at time 0) and time, with a random effect of individual. Ultrasound muscle width increased significantly with increasing M_b ($F_{1,122}$=7.06, P=0.009; Fig. 3). Muscle width also increased with acclimation time ($F_{1,122}$=7.06, P=0.009; Fig. 3). Photoperiod, temperature and the interactions temperature × time, photoperiod × time, temperature × photoperiod, and temperature × photoperiod × time were not included in the best-supported model for ultrasound muscle width.

Neither ANOVA nor ANCOVA detected significant differences among acclimation treatments in pectoralis, supracoracoideus or leg muscle masses after 6 weeks of acclimation (Fig. 4). However, heart mass did vary significantly among treatments by both ANOVA ($F_{3,39}$=8.349, P<0.001; Fig. 4) and ANCOVA ($F_{4,38}$=6.916, P<0.001). Both cold-acclimated groups had significantly larger hearts than either warm-acclimated group by ANOVA (P<0.004 in both cases), but no significant differences occurred between differing photoperiods at a given temperature. After correcting for M_b by ANCOVA, the cold SD group had significantly larger hearts than both warm SD (P=0.023) and warm LD groups (P=0.001), and the cold LD group had significantly larger hearts than the warm LD group (P=0.002), but other groups did not differ significantly. The ratio of supracoracoideus mass to pectoralis mass (S/P ratio) did not vary significantly among acclimation groups ($F_{3,39}$=1.096, P=0.362) and averaged 0.130±0.009.

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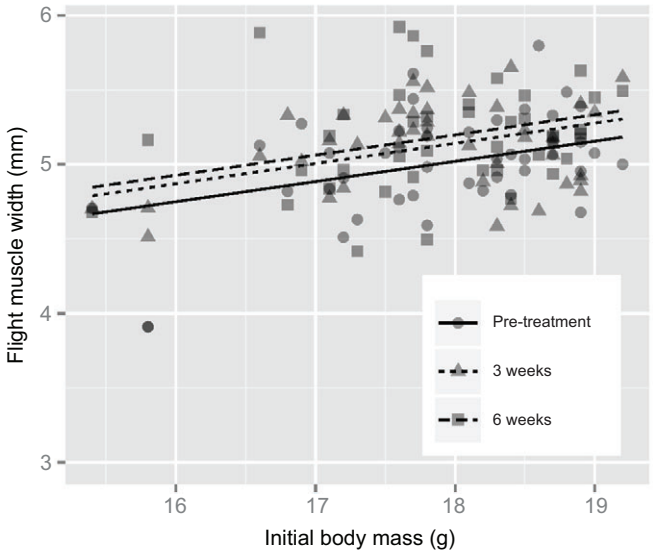

Fig. 3. Flight muscle width (mm) measured by ultrasonography as a function of initial body mass (M_b at time 0) among temperature–photoperiod acclimation groups for dark-eyed juncos. Neither temperature nor photoperiod was included in the best-supported models, but muscle width increased slightly (<5%) over the acclimation period.

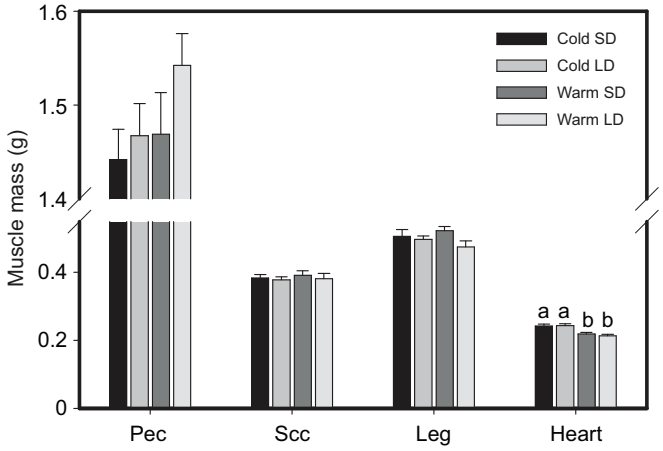

Fig. 4. Variation in mean ± s.e.m. skeletal muscle and heart masses after 6 weeks of acclimation among temperature–photoperiod acclimation groups for dark-eyed juncos. Different letters above the bars represent significant differences among acclimation groups. No significant differences were detected among acclimation groups for any of the skeletal muscles, but heart mass was significantly greater in cold-acclimated than in warm-acclimated juncos. Pec, pectoralis; Scc, supracoracoideus.

Enzyme activities

CS activity (an indicator of maximal cellular metabolic intensity) differed significantly among acclimation treatments for pectoralis muscle ($F_{3,39}$=2.864, P=0.050), but not for other muscles (Fig. 5). For pectoralis, both photoperiod and temperature appeared to influence CS activity, with cold-acclimated groups having generally higher activities than warm-acclimated groups and LD groups having generally higher activities than SD groups. However, the only significant differences in CS activity were for the warm SD group, which had significantly lower CS activity than for the cold LD (P=0.034) and warm LD (P=0.011) groups.

Carnitine palmitoyl transferase (CPT; an indicator of fatty acid transport across the mitochondrial membrane) and HOAD (an indicator of fatty acid oxidation capacity) activities demonstrated trends generally similar to those of CS activity, with significant or nearly significant differences for the pectoralis, but not for other muscles (Fig. 5). For pectoralis CPT activity, cold-acclimated groups had generally higher activities than warm-acclimated groups and LD groups had generally higher activities than SD groups, but differences did not quite attain statistical significance ($F_{3,37}$=2.663, P=0.064). CPT activity in the supracoracoideus muscle also showed a non-significant trend toward higher values in LD than in SD groups ($F_{3,37}$=2.468, P=0.077), but CPT activities in the leg and heart did not differ significantly among acclimation groups. Pectoralis HOAD activity differed significantly among acclimation treatments ($F_{3,38}$=5.622, P=0.003) with activities for the cold LD group significantly higher than for both the cold SD (P=0.002) and the warm SD (P<0.001) groups, but no other significant differences among acclimation treatments. HOAD activity did not differ significantly among acclimation treatments for the supracoracoideus, leg or heart.

DISCUSSION

The significant elevation of $\dot{M}_{sum}$ in cold-acclimated juncos, with no significant effect of photoperiod, suggests a primary role for temperature in modulating seasonal phenotypic flexibility of $\dot{M}_{sum}$ in this species. Cold-acclimated juncos in this study showed increases in $\dot{M}_{sum}$ of 16–19% after 6 weeks of acclimation. These increases are only slightly lower than the summer to winter increment of $\dot{M}_{sum}$ (28%) in free-living dark-eyed juncos from Oregon (Swanson, 1990), despite the relatively mild (3°C) and stable cold exposure. The $\dot{M}_{sum}$ increases with cold acclimation for juncos in this study also fit into the lower end of the seasonal range of $\dot{M}_{sum}$ variation for small birds in temperate-zone climates, with summer to winter increments generally in the 10–50% range (Swanson, 2010). The colder minimum temperatures and increased temperature variability encountered by wild birds in cold winter climates probably result in generally higher seasonal $\dot{M}_{sum}$ increments relative to the cold acclimation protocol in this study, as $\dot{M}_{sum}$ varies with temperature [higher during colder winters (Swanson and Olmstead, 1999; Petit et al., 2013)] and with temperature variability (Vézina et al., 2006; Vézina et al., 2011).

The prominent impact of temperature rather than photoperiod on $\dot{M}_{sum}$ for juncos in the present study is consistent with the proximate role for winter temperature on thermogenic capacity documented for small birds, including dark-eyed juncos, in cold winter climates (Swanson and Olmstead, 1999), where birds in colder winters had higher $\dot{M}_{sum}$, and short- and medium-term temperature variables (1–30 days prior to $\dot{M}_{sum}$ measurement) provided the greatest predictive power for $\dot{M}_{sum}$. Petit et al. (Petit et al., 2013) also documented inter- and intra-seasonal variation in $\dot{M}_{sum}$ in black-capped chickadees *Poecile atricapillus* consistent with a proximate role for temperature in mediating thermogenic capacity. Moreover, cold acclimation in captive red knots *Calidris canutus* over a complete annual cycle with natural photoperiod resulted in elevated $\dot{M}_{sum}$ compared with warm acclimation, and warm-acclimated birds failed to show elevated thermogenic capacity during the winter photoperiod, although $\dot{M}_{sum}$ did increase during the spring migration period (Vézina et al., 2011). Collectively, these data suggest a lesser role for photoperiod than temperature in driving seasonal metabolic variation in birds.

Cold-acclimated juncos in this study showed stepwise increases in $\dot{M}_{sum}$ over the 6-week acclimation period, with increases in $\dot{M}_{sum}$ of 9–13% after 3 weeks and 16–19% after 6 weeks of acclimation.

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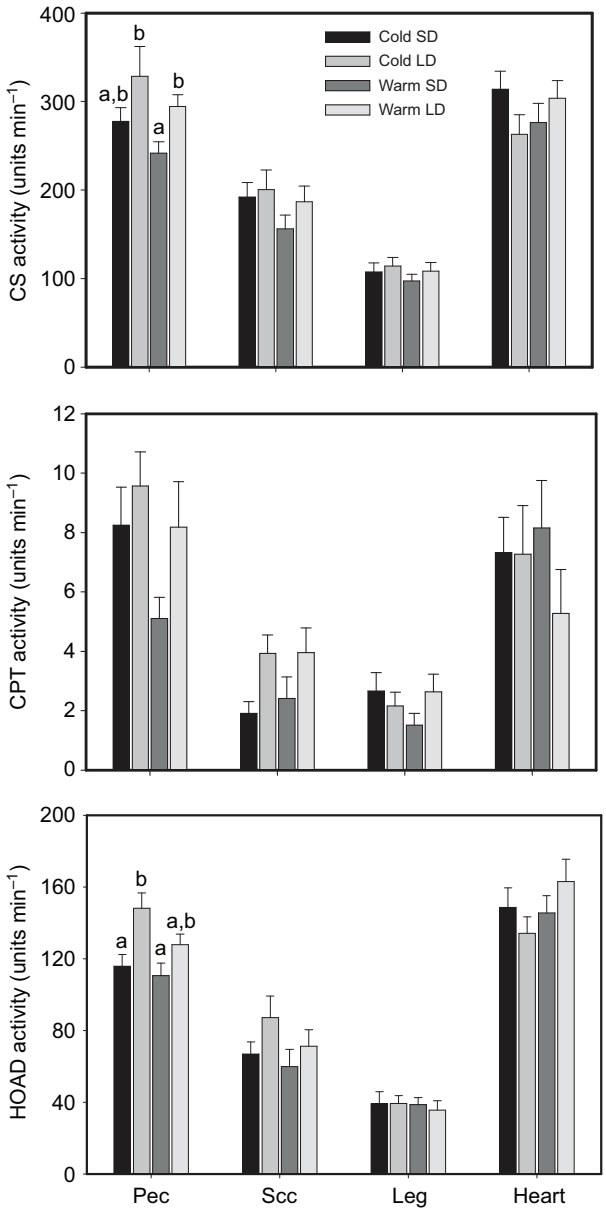

Fig. 5. Variation in mean ± s.e.m. catabolic enzyme activities in skeletal muscle and heart after 6 weeks of acclimation among temperature–photoperiod acclimation groups for dark-eyed juncos. Different letters above the bars represent significant differences among acclimation groups. No significant differences were detected for any muscle expect for pectoralis muscle, where activities for juncos on long days were generally higher than for those on short days, and cold-acclimated juncos also showed generally higher activities than warm-acclimated birds. CS, citrate synthase; CPT, carnitine palmitoyl transferase; HOAD, β-hydroxyacyl CoA dehydrogenase; Pec, pectoralis; Scc, supracoracoideus.

This indicates that the full development of the flexible metabolic response of $\dot{M}_{sum}$ to cold acclimation occurred over a longer period than 3 weeks, potentially reaching a plateau somewhere between 3 and 6 weeks. However, because we terminated the experiment at 6 weeks we cannot rule out a continued rise in $\dot{M}_{sum}$ with even longer periods of acclimation. This metabolic response to temperature appears somewhat slower than that documented for wild, free-living populations of juncos, for which temperature variables over the 14–30 day period prior to $\dot{M}_{sum}$ measurement were the best predictors of $\dot{M}_{sum}$ variation, and the single best predictor was the average minimum temperature over the 14 day period preceding $\dot{M}_{sum}$ measurement (Swanson and Olmstead, 1999). In addition, $\dot{M}_{sum}$ from juncos wintering in South Dakota decreased by up to 17% between cold and warm months within a winter, although some of this difference may be attributed to differences in M_b, as mass-specific $\dot{M}_{sum}$ decreased by only 6% for these same birds (Swanson and Olmstead, 1999). These differences in temporal responses to temperature between winter-acclimatized and cold-acclimated juncos may relate to differences in temperature exposure, as free-living birds encountered much lower minimum temperatures than cold-acclimated birds. This idea is consistent with differences in $\dot{M}_{sum}$ values, which are generally higher for free-living juncos, ranging from 6.6 to 8.4 ml O_2 min^{-1} for different winters at different locations (Swanson, 1990; Swanson, 1993; Swanson and Olmstead, 1999; Dawson and Olson, 2003; Swanson and Liknes, 2006), than for cold-acclimated juncos in this study (6.73 ml O_2 min^{-1}). Thus, although temperature appears to be a primary modulator of thermogenic capacity in small birds, the precise details of the time course of flexibility of $\dot{M}_{sum}$ in response to temperature and temperature variation, and whether such $\dot{M}_{sum}$ flexibility differs among populations (i.e. reaction norms) or with climatic variation, will require further study.

Of the skeletal muscles and heart masses examined in this study, only heart mass increased significantly with cold acclimation, and photoperiod did not significantly influence any of the muscle masses. Similar increases in both $\dot{M}_{sum}$ and heart mass in winter relative to summer for species wintering in cold climates (Swanson, 2010; Liknes and Swanson, 2011a) suggest that winter increments of heart mass are an important and general contributor to metabolic adjustments to cold climates in small birds. Moreover, these data suggest that temperature is the prominent driver of summer to winter variation in exercise organs in birds and that increases in heart mass in response to elevated thermogenic demands help drive winter increases in organismal thermogenic capacity. Heart mass is also positively correlated with maximum exercise metabolic capacity within individual house sparrows, *Passer domesticus* (Chappell et al., 1999), and red junglefowl, *Gallus gallus* (Hammond et al., 2000), suggesting that heart mass is consistently positively associated with exercise or thermogenic performance in birds.

Increases in pectoralis muscle mass are also a common component of phenotypic flexibility associated with winter or cold acclimation in many small birds (Vézina et al., 2006; Vézina et al., 2007; Swanson, 2010; Liknes and Swanson, 2011a). Nevertheless, cold acclimation in the present study did not produce significant changes in flight muscle width or significant differences in pectoralis mass after acclimation, suggesting that flight muscle hypertrophy is not a significant driver of the cold-induced increases in thermogenic capacity. This contrasts with seasonal data from many small birds wintering in cold climates, including dark-eyed juncos, where pectoralis mass increases in winter relative to summer (Swanson, 1991a; Swanson, 2010; Liknes and Swanson, 2011a). For example, pectoralis mass and $\dot{M}_{sum}$ both increased by 28% in winter for dark-eyed juncos from Oregon (Swanson, 1991a). The absence of correlated acclimation-induced variation in flight muscle masses and thermogenic capacity for juncos in the present study suggests that changes in cellular metabolic intensity and/or lipid catabolic capacity might drive the cold-induced increase in $\dot{M}_{sum}$. However, neither cellular metabolic intensity nor lipid catabolic capacity changed significantly in response to cold acclimation, although cold-acclimated birds did generally have higher catabolic enzyme activities than warm-acclimated birds (Fig. 5). This suggests that while cellular metabolic intensity and fat catabolic capacity may

The Journal of Experimental Biology (2014) doi:10.1242/jeb.096677

contribute to seasonal changes in $\dot{M}_{sum}$, they are also not strong drivers of the cold-induced increase in $\dot{M}_{sum}$ for cold-acclimated juncos in this study.

The S/P ratio did not differ significantly among acclimation groups. Seasonal variation in this ratio has been documented for red knots (Dietz and Piersma, 2007) and house sparrows (Swanson and Merkord, 2013), but this variation is not associated with seasonal variation in thermogenic capacity. Because birds shiver isometrically, with opposing muscle groups working against each other, one mechanism potentially limiting shivering thermogenesis in birds is the size of the smaller muscle, which in the case of the flight muscles is the supracoracoideus (Marsh and Dawson, 1989). The absence of similar acclimation-induced variation in $\dot{M}_{sum}$ and the S/P ratio among acclimation groups for juncos in the present study is inconsistent with such a scenario. Thus, these data support the conclusions of Liknes and Swanson (Liknes and Swanson, 2011a) and Swanson and Merkord (Swanson and Merkord, 2013) that disproportionate increases in supracoracoideus mass relative to pectoralis mass do not contribute to variation in thermogenic capacity in small birds.

Body mass typically increases in winter relative to summer and with cold acclimation for small birds in cold climates, including juncos (Blem, 1990; Swanson, 1990; Swanson, 1991b). Many of these increases involve fat stores, and seasonal differences in fat stores tend to be greatest for ground-foraging species such as dark-eyed juncos (Dawson and Marsh, 1986; Rogers and Smith, 1993; Rogers, 1995). For winter birds, this increase in fat provides a buffer to allow survival when periods of foraging might be interrupted by inclement weather (Blem, 1990). However, lean mass increases also contribute to winter and cold-induced increases in M_b, with muscle and digestive organs often increasing in winter (e.g. Zhang et al., 2008; Zheng et al., 2008; Liknes and Swanson, 2011b) or in response to cold acclimation (e.g. Vézina et al., 2006; Vézina et al., 2011). In addition, dark-eyed juncos from western Oregon showed winter increases in M_b of 8–9% (Swanson, 1990; Swanson, 1991b). Thus, we expected M_b in juncos to increase with cold acclimation in this study, but this was not the case, as temperature was not included in the best-supported models for M_b. However, LD birds showed greater M_b increments (19–20%) over the acclimation period than SD birds (4–5%). Thus, long days affected M_b more than cold acclimation for the juncos in this study.

M_b and lipid stores also increase during migration in birds, providing a fuel store for migratory flights (Blem, 1990). It has long been known that photostimulation from mid-winter through spring can increase M_b, lipid stores and migratory restlessness associated with the development of the migratory disposition in captive dark-eyed juncos (Wolfson, 1952; Eyster, 1954; Farner et al., 1961; Johnston, 1962). Holberton et al. (Holberton et al., 2007; Holberton et al., 2008) photostimulated captive juncos in mid-winter or early spring and found that M_b and fat stores increased rapidly upon photostimulation, with significant increases within 5–15 days and continued elevation for the duration of the 29–47 day photostimulation treatments. Thus, the increase in M_b for LD juncos in the present study seems likely to be related to the development of migratory disposition in these birds. The M_b increase on LD was only partially due to gonadal growth (accounting for ~10% of M_b gains; authors' personal observations) and was not due to flight muscle hypertrophy, so other body constituents must have also contributed. Lean mass changes also occur during migration and muscles and digestive organs are remodeled extensively during periods of migratory flights and stopover (Piersma and van Gils, 2011). We did not measure carcass fat or masses of digestive organs in this study, but these tissues are likely candidates for promoting the increase in M_b on LD. Although we did not measure carcass fat directly, LD birds did appear to carry more fat than SD birds authors' personal observations), consistent with expectations for birds in a migratory disposition.

How such changes in M_b relate to the differences in $\dot{M}_{sum}$ among treatment groups, with higher $\dot{M}_{sum}$ values in cold-acclimated birds, is uncertain. Perhaps the generally increased catabolic enzyme capacities noted for LD relative to SD juncos were also related to the development of migratory disposition and adjustment for endurance flight, as cellular metabolic intensity and fat catabolic capacity increase in some, though not all, migratory birds (Dawson et al., 1983; Swanson, 2010). For example, neither muscle lipoprotein lipase nor adipose tissue lipolytic capacity varied between winter, early spring and migratory periods for outdoor captive dark-eyed juncos from New York, despite marked differences in M_b and fat stores, although muscle lipoprotein lipase activity did increase at night during periods of migratory restlessness (Ramenofsky et al., 1999). Also germane to this discussion, photostimulation, which induced migratory restlessness in white-crowned sparrows (*Zonotrichia leucophrys*), failed to increase pectoralis muscle activities of CS, CPT or HOAD or fat transporter expression, suggesting that actual migratory flights are required for elevations of cellular metabolic intensity and fat catabolic capacity in this species (Price et al., 2010). Higher M_b and pectoralis cellular metabolic intensity for LD juncos in our study was not associated with variation in organismal $\dot{M}_{sum}$, although an increase in $\dot{M}_{sum}$ with migratory condition has been demonstrated in several studies of migratory birds (Swanson, 1995; Swanson and Dean, 1999; Vézina et al., 2006; Vézina et al., 2007). However, because maximum exercise metabolic rate and $\dot{M}_{sum}$ are sometimes positively associated among birds, but oftentimes not (Wiersma et al., 2007; Swanson et al., 2012), the absence of positive correlations between M_b, catabolic enzyme activities and $\dot{M}_{sum}$ in the present study does not necessarily eliminate the possibility of a relationship between M_b, catabolic enzyme activities and exercise metabolic capacity associated with migratory disposition.

Cellular metabolic intensity and fat catabolic capacity in the pectoralis and other skeletal muscles are variably related to winter increases in $\dot{M}_{sum}$ in small birds. Cellular metabolic intensity of pectoralis muscle, inferred from CS or COX activities, is higher in winter than in summer for several bird species (Vézina and Williams, 2005; Zheng et al., 2008; Zheng et al., 2010; Liknes and Swanson, 2011b), but is seasonally stable for several others (Marsh and Dawson, 1982; Yacoe and Dawson, 1983; Carey et al., 1989; O'Connor, 1995; Liknes and Swanson, 2011b; Sgueo et al., 2012). Similar variability in seasonal trends for cellular metabolic intensity occurs in leg muscles (Carey et al., 1989; Marsh and Dawson, 1982; Liknes and Swanson, 2011b). Fat catabolism capacity in the pectoralis and/or leg muscles also increases in winter for some species, but remains seasonally stable in others (Marsh and Dawson, 1982; Yacoe and Dawson, 1983; Carey et al., 1989; O'Connor, 1995; Liknes and Swanson, 2011b). Likewise, cellular metabolic intensity and fat catabolic capacity vary with migratory condition for some species, but not for others (Dawson et al., 1983; Swanson, 2010), even though migration regularly increases $\dot{M}_{sum}$ (Swanson, 1995; Swanson and Dean, 1999; Vézina et al., 2006; Vézina et al., 2007). Collectively, these data highlight the variable nature of seasonal patterns of catabolic enzyme activities in small birds and the species-specific nature of the contribution of such variation to metabolic phenotypes. In addition, evidence as to whether responses to temperature, exercise or photoperiod drive seasonal variation in

cellular metabolic intensity or lipid catabolic capacity, if such variation is present, is currently equivocal.

In summary, temperature appears to be the primary driver of organismal variation in thermogenic capacity in juncos, and this elevated $\dot{M}_{sum}$ was associated with higher heart, but not skeletal muscle, masses in this study. In contrast, M_b of juncos responded more strongly to photoperiod than temperature, and in a manner consistent with development of migratory disposition, gonad growth and other reproductive changes. Photoperiod and temperature both affected pectoralis muscle cellular metabolic intensity in juncos, although photoperiod responses were more prominent. Thus, these data suggest that (1) the interplay between temperature and photoperiod to generate flexible phenotypes is complex, (2) adjustments promoting cold tolerance and migratory flight may interact, and (3) exposure to synthetic environmental cues in captivity may not fully mimic natural acclimatization in wild birds.

MATERIALS AND METHODS

Bird capture and acclimation

We captured juncos near Vermillion, South Dakota, during mid-December, early in the winter period. We housed birds individually in 59×45×36 cm stainless-steel cages in animal rooms with temperature (±2°C) and photoperiod control and allowed them to adjust to captive conditions, at room temperature (23°C) and normal photoperiod for the time of capture, for at least 2 weeks, after which body mass stabilized (authors' personal observations), before we initiated acclimation treatments. Following the initial 2 week captivity-adjustment period, we acclimated birds (n=12 birds for each treatment group) to four temperature–photoperiod treatments, each of 6 weeks in duration. The treatment groups were 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). While in captivity, we provided birds with seed (millet and black oil sunflower seed), a protein supplement (consisting of a homogenized mixture of dry dog food, with a minimum protein content of 21%, and hard-boiled eggs), and vitamin-enriched (Wild Harvest Multi-Drops vitamin supplement for all birds, United Pet Group, Inc., Cincinnati, OH, USA) water *ad libitum*.

We measured M_b, flight muscle thickness by ultrasound, and $\dot{M}_{sum}$ before acclimation treatments and at 3 and 6 weeks of acclimation. For these variables, we measured $\dot{M}_{sum}$ 1 day prior to ultrasound measurements to eliminate the potential for feather matting from the ultrasound gel influencing cold tolerance capacity during $\dot{M}_{sum}$ measurement. Following the 6 week acclimation treatments, we euthanized juncos by cervical dislocation and measured several variables related to phenotypic flexibility in the pectoralis, supracoracoideus and leg muscles, and heart. These measurements included body and muscle masses and enzyme activities for CS (EC 4.1.3.7), CPT (EC 2.3.1.21) and HOAD (EC 1.1.1.35). After euthanasia, we rapidly excised muscles on ice, weighed them to the nearest 0.1 mg, and snap-froze tissues in liquid nitrogen. Frozen tissues were maintained at −80°C until later enzyme assays.

We collected birds under appropriate state (11-7, 12-2) and federal (MB758442) scientific collecting permits and all procedures reported herein were approved by the University of South Dakota Institutional Animal Care and Use Committee (Protocol 79-01-11-14C).

$\dot{M}_{sum}$ measurement

We measured thermogenic performance as $\dot{M}_{sum}$ by open-circuit respirometry in an atmosphere of 21% oxygen/79% helium (helox) using a sliding cold exposure (Swanson et al., 1996). Helox facilitates heat loss so that $\dot{M}_{sum}$ can be attained at relatively moderate temperatures without impairing oxygen uptake (Rosenmann and Morrison, 1974; Holloway and Geiser, 2001; Arens and Cooper, 2005). For the sliding cold exposure protocol, we first flushed the metabolic chamber with helox for 5 min prior to initiation of cold exposure to replace chamber air with helox. After this period, we initiated the cold exposure by placing the metabolic chamber into the anti-freeze bath. The initial temperature in helox ranged from 3 to −5°C depending on the size of the bird (lower temperatures for larger birds). For the initial 20 min of cold exposure, we kept the bath temperature at the starting temperature, after which we reduced bath temperature by ~3°C every 20 min. We continued the sliding cold exposure treatment until we detected a steady decline in oxygen consumption over several minutes, indicative of hypothermia. At this time we removed birds from the metabolic chamber and recorded body temperature with a thermocouple thermometer by inserting a lubricated 20 gauge thermocouple into the cloaca to a depth of ~0.1 cm. We considered body temperatures of ≤37°C as hypothermic and all birds were hypothermic at the end of cold exposure trials, which validated that $\dot{M}_{sum}$ had been attained. We weighed birds to the nearest 0.1 g both before and after $\dot{M}_{sum}$ measurements and assumed constant mass loss over the measurement period. The sliding cold exposure in this study generally lasted for approximately 30–90 min for individual birds.

The open-circuit respirometry system used metabolic chambers designed from 1.9 liter paint cans with the inside painted flat black to minimize reflection of heat produced by the bird back into the chamber. We regulated chamber temperature by immersing the chamber into an ethylene glycol bath (Forma Scientific Model 2095, Marietta, OH, USA) capable of regulating temperature to ±0.2°C. We measured oxygen content in excurrent gas from the metabolic chamber with an Ametek Model S-3AII oxygen analyzer (Pittsburgh, PA, USA) at 5 s intervals using Datacan 5.0 data collection software (Sable Systems, Henderson, NV, USA). During $\dot{M}_{sum}$ measurements, we maintained flow rates of dry, CO_2-free helox at 1010–1030 ml min^{-1} with a Cole-Parmer Precision rotameter (model FM082–03ST, Chicago, IL, USA) calibrated to ±1% accuracy with a soap bubble meter. From these data, we calculated 5 min running means of instantaneous oxygen consumption (Bartholomew et al., 1981) over the entire test period using Expedata 2.0 software (Sable Systems), and considered the highest 5 min mean oxygen consumption over the test period as $\dot{M}_{sum}$ (Wiersma et al., 2007; Swanson et al., 2012). We corrected all values for oxygen consumption to STPD.

Ultrasound measurements of muscle size

We conducted ultrasound measurements of flight muscle thickness with a Visual Sonics Vevo 770 High-Resolution Imaging System and Model 704 scan head (Toronto, ON, Canada) using a frequency of 25 MHz and focal length of 15 mm for all measurements (Swanson and Merkord, 2013; Swanson et al., 2013). We did not anesthetize birds for ultrasound measurements, but placed a cloth bag over their head to calm them yet allow breathing. We added water-soluble recording gel to the skin in the breast region and lowered the scan head into the recording gel for measurement of muscle width. We measured flight muscle width to the nearest 0.01 mm along the short axis of the flight muscles (Dietz et al., 1999; Swanson and Merkord, 2013; Swanson et al., 2013). We standardized the position for these measurements by placing a metal ruler across the bottom of the furculum and sliding the scan head forward along the musculature of the right breast so that it just touched the ruler. We measured flight muscle width along a right angle to the horizontal sternum at its junction with the sternal keel (Swanson and Merkord, 2013). We repeated this measurement four to six times on each individual bird, completely removing the scan head from the recording gel between successive measurements. All measurements were completed within 2 min for each individual bird. We used the average of these measurements, after removing any obvious outliers [>20% different from other measurements (Swanson and Merkord, 2013)], for flight muscle width in subsequent analyses. We calculated repeatability for flight muscle width measurements as the intraclass correlation coefficient (Lessells and Boag, 1987).

Enzyme activities

We measured CS activity in muscles as an indicator of maximal cellular metabolic intensity (Marsh, 1981; O'Connor, 1995; Liknes and Swanson, 2011b). After removal of muscle samples from storage at –80°C, we minced small samples and weighed them to the nearest 0.1 mg. We then mixed muscle samples in 10–40 volumes/mass of homogenizing buffer (100 mmol l^{-1} phosphate buffer with 2 mmol l^{-1} EDTA at pH 7.3) and homogenized the diluted samples on ice with a Tekmar Model ST-1810 Tissuemizer (Cincinnati, OH, USA). We then sonicated samples on ice for three 10 s bursts with 30 s between bursts with a Cole-Parmer 4710 Series

Ultrasonic Homogenizer. The assay medium contained 100 mmol l^{-1} triethanolamine-HCl, 2.5 mmol l^{-1} EDTA, 0.1 mmol l^{-1} 5.5′-dithiobis-(2-nitrobenzoic acid), 0.2 mmol l^{-1} acetyl-CoA and 0.5 mmol l^{-1} oxaloacetate (omitted for control) at pH 7.5 in a final volume of 1.0 ml (Liknes and Swanson, 2011b).

We measured the activity of CPT to assess fatty acid transport capacity across mitochondrial membranes, as palmitoyl CoA exhibited higher activity than oleoyl CoA in pilot studies of substrate affinity, similar to results from Guglielmo et al. (Guglielmo et al., 2002). We homogenized (1:10 w:v) muscles on ice in 10 mmol l^{-1} Tris buffer, containing 1 mmol l^{-1} EDTA at pH 7.5 (Driedzic et al., 1993), and then sonicated samples as for CS assays. We performed assays on crude muscle homogenates in 50 mmol l^{-1} Tris assay buffer with 0.15 mmol l^{-1} 5,5′-dithiobis-(2-nitrobenzoic acid), 5 mmol l^{-1} carnitine (omitted for control) and 0.035 mmol l^{-1} palmitoyl Co-A at pH 8.0 in a final volume of 1.0 ml (Guglielmo et al., 2002).

We measured the activity of HOAD as an indicator of fatty acid oxidation capacity (O'Connor, 1995; Liknes and Swanson, 2011b). For homogenization, we diluted muscle samples to 10–40 volumes/mass of homogenizing buffer containing 100 mmol l^{-1} phosphate and 2 mmol l^{-1} EDTA at pH 7.3 and homogenized samples on ice. Following homogenization, we sonicated homogenates as for CS assays. We assayed HOAD activity according to the methods of Bass et al. (Bass et al., 1969), as modified by Marsh (Marsh, 1981), and as previously conducted in our laboratory (Liknes and Swanson, 2011b; Swanson et al., 2013). The HOAD assay medium contained 100 mmol l^{-1} triethanolamine-HCl, 5 mmol l^{-1} EDTA, 0.225 mmol l^{-1} $NADH_2$ and 0.1 mmol l^{-1} acetoacetyl-CoA (omitted for control) at pH 7.0 in a final volume of 1.0 ml.

We conducted enzyme assays at 39±2°C with a Beckman DU 7400 spectrophotometer (Beckman Coulter, Inc., Fullerton, CA, USA). For all assays, we recorded 2–3 min of readings before adding the reaction substrate to establish control activity values. We followed reactions for 5 min after the addition of substrate for enzyme activity measurements. We then subtracted control values (if consistently different from zero) from activity values following substrate addition to calculate enzyme activities. We ran all samples in duplicate and used average values for subsequent calculations. For enzyme activity calculations, we used millimolar extinction coefficients of 13.6 at 412 nm for CS and CPT and 6.22 at 340 nm for HOAD. We report all enzyme activities as mean mass-specific activity (μmol min^{-1} g^{-1}).

Statistics

We report values as means ± s.e.m., unless otherwise noted. To test for differences among treatment groups at the beginning of the acclimation period, we used one-way ANOVA for M_b and ANCOVA with M_b as a covariate for $\dot{M}_{sum}$ and ultrasound muscle width. We $\log_{10}$-transformed $\dot{M}_{sum}$ and M_b data prior to all analyses of $\dot{M}_{sum}$.

We estimated the effects of treatment and acclimation time period on M_b, $\dot{M}_{sum}$ and ultrasound flight muscle width in a mixed-model framework using the model selection protocol outlined by Zuur et al. (Zuur et al., 2009). For each response variable, we started with a linear regression model that contained a three-way interaction effect between temperature (warm or cold), photoperiod (long day or short day) and acclimation time period (0, 3 or 6 weeks). For $\dot{M}_{sum}$ and ultrasound muscle width, we also included M_b at the time of capture as a predictor because M_b is known to influence metabolic rates and organ and muscle masses in birds (Packard and Boardman, 1988; Packard and Boardman, 1999; Hayes and Shonkwiler, 1996). We investigated homogeneity assumptions by plotting standardized residuals versus fitted values and by plotting standardized residuals versus individual explanatory variables. If visual inspection of the results indicated heterogeneity then we fit a new model with a variance–covariance structure allowing heterogeneity to vary either per stratum or as a function of M_b, compared the two models using a likelihood ratio test, and retained the best model. We then added a term for a random effect of individual to account for correlation over time, compared this model with the former by likelihood ratio test, and retained the best model. Next, we created a nested submodel by removing the least significant fixed term, selected the best model using a likelihood ratio test, and repeated this process until the model with the simplest fixed structure was identified that outperformed its submodels. We used α=0.05 to evaluate all likelihood ratio tests. We analyzed mixed-effects models in R (R Development Core Team, 2013) using the nlme package (Pinheiro et al., 2013) and conducted other statistical tests with SigmaStat version 3.5 (Systat Software, Inc., Port Richmond, CA, USA).

For muscle mass comparisons at the end of the acclimation period, we used both one-way ANOVA and ANCOVA, with M_b minus the mass of the muscle (including both sides of the muscle if the muscle was paired) for the body mass covariate to avoid problems with part–whole correlations (Christians, 1999). We used both statistical tests for these comparisons to account for potential differences in body fat among acclimation groups, which might contribute to differences in M_b, but little to metabolic differences. We used one-way ANOVA to test for differences among treatment groups for enzyme activities. We used Fisher's least significant difference as a *post hoc* test to identify which groups differed with ANOVA. For ANCOVA *post hoc* tests, we ran Tukey's all-pairwise comparisons in R using the multcomp package (Hothorn et al., 2008).

We $\log_{10}$-transformed data prior to ANOVA or ANCOVA when parametric assumptions were not met (usually non-normal distribution), which occurred for a few muscle measurements and several enzyme activities in different muscles. To test for correlations between ultrasound measures of flight muscle width and flight muscle mass from dissections, we used least squares linear regression. We accepted statistical significance at $P \leq 0.05$.

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Competing interests

The authors declare no competing financial interests.

Author contributions

D.S. and M.K. conceived the study and designed the experiments; D.S., Y.Z., J.-S.L. and M.K. collected the data; D.S. and C.M. analyzed the data; D.S. wrote the manuscript; D.S., Y.Z., J.-S.L. C.M. and M.K. interpreted data and revised the manuscript. All authors assume responsibility for the content of the paper.

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