Cross-training in birds: cold and exercise training produce similar changes in maximal metabolic output, muscle masses and myostatin expression in house sparrows, *Passer domesticus*

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Abstract
Maximal metabolic outputs for exercise and thermogenesis in birds presumably influence fitness through effects on flight and shivering performance. Because both summit ($M_{\text{sum}}$ = maximum thermoregulatory metabolic rate) and maximum (MMR = maximum exercise metabolic rate) metabolic rates are functions of skeletal muscle activity, correlations between these measurements and their mechanistic underpinnings might occur. To examine whether such correlations occur, we measured the effects of 3-week experimental cold and exercise training protocols on body ($M_b$) and muscle masses, basal metabolic rate (BMR), $M_{\text{sum}}$, MMR, pectoralis mRNA and protein expression for myostatin, and mRNA expression of TLL-1 and TLL-2 (metalloproteinase activators of myostatin) in house sparrows (*Passer domesticus*). Both training protocols increased $M_{\text{sum}}$, MMR, $M_b$, and pectoralis mass, but BMR increased with cold- and decreased with exercise-training. No significant differences occurred for pectoralis myostatin mRNA expression, but cold and exercise promoted higher TLL-1 and TLL-2 expression. Pectoralis myostatin protein levels were generally reduced for both training groups. These data clearly demonstrate cross-training effects between cold and exercise in birds and are consistent with a role for myostatin inducing increased pectoralis muscle mass and driving organismal increases in metabolic capacities.

**Key Words:** Phenotypic flexibility, cold training, exercise training, pectoralis, myostatin, MMR, $M_{\text{sum}}$, BMR, birds

**Summary statement**
Cold and exercise training in house sparrows increased thermogenic and exercise capacities and pectoralis masses and modified expression of the myostatin system consistent with a mechanistic role for myostatin down-regulation.
Introduction

Ecological and evolutionary physiologists have used standardized measures of upper and lower limits of metabolic output to study the relationships among organismal physiology, performance and fitness, as well as environmental influences on these relationships (Hayes and Chappell, 1990; Piersma and Van Gils, 2010; Bozinovic et al., 2011). Formerly, many of these studies used single trait values for a species as representative of species-environment relationships, but the degree of flexibility in these traits within individuals has recently become more appreciated (Seebacher, 2005; McKechnie, 2008; Swanson, 2010). Reversible phenotypic flexibility allows animals to better match their phenotypes to changing ecological and energetic demands (Piersma and Drent, 2003), and such changes are often manifested by changing upper and lower limits of metabolic output (e.g., McKechnie, 2008; Swanson 2010). Metabolic outputs for a variety of endothermic species have been studied during different performance functions, especially locomotion and cold exposure, and metabolic outputs are generally upregulated under conditions of increasing energy demands (Dawson and Marsh, 1989; Weibel and Hoppeler, 2005; Swanson, 2010).

Both shivering thermogenesis and flight in birds are functions of skeletal muscle activity and share similar metabolic pathways and substrates (Marsh and Dawson, 1982; Wiersma et al., 2007; Guglielmo, 2010). Consequently, correlations between maximum metabolic rates during exercise (MMR) and cold (summit metabolic rate, M_{sum}) might be expected both among and within species (Swanson, 2010). However, only a few studies have examined relationships among basal metabolic rate (BMR, lower limit of metabolic output), MMR, and M_{sum} (Wiersma et al., 2007; Swanson et al., 2012; Petit et al., 2013; Careau et al., 2014) and these studies fail to show consistent correlations among BMR, MMR, and M_{sum}. To our knowledge, no studies have explicitly examined cross-training effects (i.e., exercise-training effects on shivering performance and cold-acclimation effects on exercise performance) on the upper limits of metabolic performance produced by both exercise and cold in birds, although Petit and Vézina (2014) found that experimentally increasing flight costs improved shivering performance in black-capped chickadees (Poecile atricapillus). Because of the shared structures (skeletal muscles) and fuel and oxygen supply pathways for exercise and shivering in birds, similar adjustments could produce cross-training effects on organismal performance, and such effects could have fitness consequences. For example, spring migrant birds with elevated capacities for flight metabolism could improve thermogenic performance as a by-product, which could be beneficial for thermoregulation as they encounter cold temperatures during migration or upon arrival at the breeding grounds (Swanson and Dean, 1999; Vézina et al., 2007; Corder and Schaeffer, 2015).
Skeletal muscle mass, especially pectoralis mass, is positively related to maximal metabolic output for many bird species (Chappell et al., 1999; Swanson, 2010; Petit et al., 2013; Swanson and Merkord, 2013; Swanson et al., 2013; Petit and Vézina, 2014; Swanson et al., 2014b). A candidate for flexible regulation of skeletal muscle mass, and thereby maximal metabolic output, 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 and McPherron, 2001; Amthor et al., 2002). Myostatin is secreted as an inactive latent form which requires cleavage by metalloproteinases, including the tolloid-like proteins TLL-1 and TLL-2, to produce the active C-terminal dimer (Huet et al., 2001; Wolfman et al., 2003). Several studies have examined variation in expression of myostatin and the TLLs in birds during periods (migration and winter acclimatization) producing variation in muscle mass (Swanson et al., 2009; Price et al., 2011; Swanson et al., 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 negative relationships between expression of myostatin and the TLLs and pectoralis muscle mass (Swanson et al., 2009), others finding no or positive relationships (Price et al., 2011), and still others finding different trends for mRNA and protein levels (Swanson et al., 2014a; King et al., 2015). Nevertheless, the negative relationships among skeletal muscle masses and myostatin or TLL expression for some species, including the focal species for this study, house sparrows Passer domesticus (Swanson et al., 2009), are consistent with a role for myostatin in mediating metabolic flexibility in these species.

Several studies have examined cross-training effects of exercise or cold on maximal metabolic outputs in mammals. In general, cold acclimation increases MMR (Turner et al., 1995; Florez-Duquet and McDonald, 1998) and exercise training increases heat production and $M_{\text{sum}}$ (McDonald et al., 1988; Shechtman and Talan, 1994) in mammals. Similar experiments monitoring cross-training effects on maximal metabolic outputs for exercise and thermogenesis are less common for birds, despite the primary role of skeletal muscle in generating both $M_{\text{sum}}$ and MMR because of the lack brown fat in birds (Cannon and Nedergaard, 2004; Mezentseva et al., 2008).

In the present study, we used house sparrows as our study species, as they exhibit winter increases in $M_{\text{sum}}$ and pectoralis muscle mass along with winter decreases in myostatin and TLL-1 mRNA expression (Arens and Cooper, 2005; Swanson and Liknes, 2006; Swanson et al., 2009; Liknes and Swanson, 2011; Swanson and Merkord, 2013). We employed experimental acute cold and exercise training protocols to modify phenotypes of birds and measured $M_{b}$, BMR, $M_{\text{sum}}$, and MMR before and after training treatments. We also measured skeletal muscle and heart masses,
as well as pectoralis mRNA expression of myostatin and the TLLs, and protein levels of latent and active myostatin to examine potential mechanistic roles for muscle masses and the myostatin system in regulating training-induced metabolic flexibility. We hypothesized that: 1) cross-training effects occur in birds, such that cold training will increase exercise capacity and exercise training will increase thermogenic capacity; 2) both training protocols will result in increased flight muscle and heart masses to support elevated aerobic capacities; and 3) myostatin, TLL-1, and TLL-2 expression will be lower for exercise- and cold-trained birds relative to controls. To our knowledge, this study is the first to directly test for cross-training effects between exercise and cold in birds and thereby test assumptions about potential fitness consequences of metabolic adjustments produced as by-products of selection for exercise or thermogenic capacities.

Results

Cold and Exercise Training Effects on Metabolic Rates

Pre-treatment measurements

ANCOVA detected no significant differences in intercepts of allometric regressions between cold-trained and control groups for pre-training BMR ($F_{1,15} = 0.36$, $P = 0.560$), $M_{sum}$ ($F_{1,17} = 0.73$, $P = 0.405$), or MMR ($F_{1,13} = 0.44$, $P = 0.520$) measurements. Neither were significant differences in intercepts detected for pre-training BMR ($F_{1,15} = 1.79$, $P = 0.204$), $M_{sum}$ ($F_{1,15} = 2.11$, $P = 0.171$), or MMR ($F_{1,15} = 2.59$, $P = 0.131$) measurements between exercise-trained and control groups.

Exercise-trained birds showed significantly higher MMR (16.5%, $F_{1,13} = 6.59$, $P = 0.026$), with a non-significant trend toward higher $M_{sum}$ (11%, $F_{1,16} = 3.43$, $P = 0.085$), than cold-trained birds. Exercise-training control birds also had significantly higher $M_{sum}$ (11.9%, $F_{1,16} = 5.59$, $P = 0.033$) and MMR (17.9%, $F_{1,14} = 18.3$, $P = 0.001$) than cold-training control birds.

Post-treatment measurements

ANCOVA revealed significantly higher values in cold-trained than in control birds for $M_{sum}$ ($F_{1,17} = 5.55$, $P = 0.033$) and MMR ($F_{1,12} = 16.49$, $P = 0.002$) (Figure 1). A non-significant trend toward higher BMR ($F_{1,16} = 3.75$, $P = 0.073$) in cold-trained birds was also evident. Within the cold-trained birds there were significant increases in both $M_{sum}$ (14.6%, $F_{1,18} = 9.41$, $P = 0.008$) and MMR (12.1%, $F_{1,12} = 8.63$, $P = 0.017$), with a similar non-significant trend for BMR (10.3%, $F_{1,16} = 3.85$, $P = 0.072$), between pre- and post-training measurements, whereas no
significant differences were found for control birds. For post-exercise-training measurements, we
found significantly higher values for $M_{\text{sum}}$ ($F_{1,14} = 5.77, P = 0.033$) and MMR ($F_{1,15} = 21.72, P < 0.001$), with significantly lower values for BMR ($F_{1,15} = 9.07, P = 0.010$), in exercise-trained
birds relative to controls (Figure 2). After training, exercise-trained birds also showed
significantly elevated $M_{\text{sum}}$ (15.5%, $F_{1,16} = 5.46, P = 0.036$) and MMR (19.7%, $F_{1,16} = 14.39, P = 0.002$) and a significantly lower BMR (37.9%, $F_{1,16} = 11.63, P = 0.005$) relative to pre-training
measurements. Exercise-training control birds did not exhibit significant temporal changes.

Exercise-trained birds showed significantly higher $M_{\text{sum}}$ (11.6%, $F_{1,16} = 4.77, P = 0.047$)
and MMR (24.4%, $F_{1,13} = 31.75, P < 0.001$) compared to cold-trained birds. Between post-
training control groups, exercise-training control birds had significantly higher $M_{\text{sum}}$ (12.9%, $F_{1,15} = 5.97, P = 0.029$) and MMR (18.7%, $F_{1,14} = 42.78, P < 0.001$) compared to cold-training control
birds. Cold-trained birds showed a non-significant trend toward higher (14.9%, $t_{12} = 2.21, P = 0.061$) post-training $M_{\text{sum}}$/MMR ratios (0.68 ± 0.09) compared to exercise-trained birds (0.59 ± 0.07), whereas no differences in $M_{\text{sum}}$/MMR ratios occurred between cold- (0.60 ± 0.06) and
exercise-trained (0.59 ± 0.05) control groups.

Body and Tissue Masses

There was no difference in pre-training $M_b$ between either training group and their
associated control groups (Table 1). However, both cold- (8%, $t_{16} = 2.21, P = 0.04$) and exercise-
trained (7%, $t_{14} = 2.694, P = 0.017$) groups showed significantly higher $M_b$ than controls for post-
training measurements. In addition, post-training body mass was higher than pre-training mass by
7% ($t_{16} = 2.73, P = 0.015$) in cold trained birds and by 6.5% ($t_{14} = 4.122, P = 0.001$) in exercise
trained birds, whereas there was no change in either control group (Table 1). Pectoralis muscle,
but not heart, was significantly larger ($F_{1,17} = 5.76, P = 0.030$) in cold-trained than in control
birds (Figure 3). Exercise-trained birds showed non-significant trends toward increases in both
pectoralis muscle ($F_{1,15} = 4.13, P = 0.063$) and heart ($F_{1,15} = 3.64, P = 0.079$) masses relative to
control birds (Figure 3).

mRNA Expression and Protein Levels

Pectoralis myostatin mRNA expression did not differ significantly between cold-trained
and control birds, despite a 3.7-fold higher average expression in control than in cold-trained
groups (Figure 4, $U_{18} = 26.0, P = 0.216$). Pectoralis mRNA expression for both TLL-1 (2.03-fold,
$t_{15} = 2.437, P = 0.028$) and TLL-2 (2.32-fold, $t_{15} = 3.931, P = 0.001$) was significantly higher in
cold-trained than in control birds. For exercise training, there were no significant differences between exercise-trained and control birds for myostatin mRNA expression ($t_{14} = 1.047, P = 0.313$). However, similar to cold training, both TLL-1 (2.2-fold, $t_{13} = 1.854, P = 0.087$) and TLL-2 (2.35-fold, $u_{15} = 44, P = 0.072$) mRNA expression showed non-significant trends toward higher values in exercise-trained than in control birds.

We detected two bands for the myostatin antibody in our Western blots, which correspond to the 52 kDa unprocessed latent form of myostatin and the 26 kDa cleaved C-terminal dimer active form. Our quantifications therefore include measurements for both inactive and active forms of myostatin. Protein levels of latent myostatin were significantly higher (1.37-fold, $t_{16} = 2.238, P = 0.04$) in control than in cold-trained birds. Similarly, protein levels for the active form of myostatin showed a non-significant trend (1.26-fold, $t_{16} = 1.93, P = 0.072$) toward higher levels in control than in cold-trained sparrows (Figure 4). For exercise-trained birds, both active (1.46-fold, $t_{14} = 2.768, P = 0.015$) and latent (1.55-fold, $t_{14} = 3.099, P = 0.008$) forms of myostatin showed lower values than in control birds (Figure 4).

**Correlations**

We detected no significant correlations for whole-organism or mass-independent (multiple regression with $M_b$ as a covariate) BMR with whole-organism or mass-independent $M_{\text{sum}}$ or MMR, either within each experimental group or for all sparrows pooled. When we pooled data for all birds, significant positive correlations were detected between whole-organism ($r^2 = 0.318, P = 0.002$) and mass-independent ($r^2 = 0.299, P = 0.012$) $M_{\text{sum}}$ and MMR. Latent myostatin protein levels were significantly negatively correlated with mass-independent $M_{\text{sum}}$ ($r^2 = 0.275, P = 0.007$). However, no significant correlation was detected for active myostatin protein levels with any metabolic rate measurement. mRNA expression and protein levels were positively correlated for latent myostatin protein ($r^2 = 0.417, P < 0.001$), but not for active myostatin protein ($r^2 = 0.02, P = 0.412$), for all birds pooled. There were no significant correlations between mRNA expression and protein levels of myostatin within any of the four experimental groups.

**Discussion**

Our acute cold-training protocol increased thermogenic capacity of sparrows, similar to cold acclimation protocols in other bird studies (McKechnie, 2008; Swanson, 2010). $M_{\text{sum}}$ increased by 14.6% in cold-trained birds relative to pre-treatment values and was 12.7% higher than in controls. These values are similar to percent changes produced by cold acclimation in
previous studies (Vézina et al., 2006; van de Ven et al., 2013b; Swanson et al., 2014b) and within the range (generally 10-50%) reported for winter acclimatization in small birds (Swanson, 2010). Exercise-trained sparrows in this study showed elevated MMR, with increases of 19.7% relative to pre-treatment birds and 14.2% compared to control birds. Studies of links between exercise training and maximum metabolic outputs are rare for birds, but this subject has been intensively studied in mammals (Thompson et al., 2012; Holloszy and Booth, 1976 for review). Exercise training generally increases mammalian MMR by 4-23% (Holloszy and Booth, 1976; Pica and Brooks, 1982; Gleeson et al., 1983; Evans and Rose, 1988; Carter et al., 2000). Similarly, exercise training produces increases of 16-28% in reptiles (Owerkowicz and Baudinette, 2008; Eme et al., 2009). Among birds, tufted ducks Aythya fuligula showed a 27% increase in MMR during swimming after swim-training (Butler and Turner, 1988).

Cross-training effects between exercise and cold were observed for sparrows in this study, suggesting that similar mechanisms underlie adjustments for both exercise, which produces changes in muscle length, and isometric shivering, which doesn’t, and that flexible responses to cold or exercise may increase performance for alternate muscular activities as a by-product. Such cross-training effects could be important to organismal ecology and have fitness consequences (Swanson and Dean, 1999; Vézina et al., 2007). For example, physiological adjustments promoting a higher thermogenic capacity are not only beneficial to cold tolerance but also could allow a higher exercise capacity for locomotion, feeding and avoiding predators, which could be beneficial in cold winter conditions where energy demands and foraging requirements also increase. Conversely, elevated physiological adjustments producing higher exercise capacity may also promote increased cold tolerance, which may be beneficial during migration, especially spring migration when birds moving to higher latitudes are likely to encounter cold temperatures. Consistent with this idea, birds typically show elevated M_sum during the spring migratory period compared with non-migratory periods (Vézina et al., 2007; Swanson, 2010; Corder and Schaeffer, 2015) and phenotypic manipulations increasing flight costs also increased M_sum in black-capped chickadees (Petit and Vézina, 2014).

Two hypotheses have been proposed to account for the phenomenon of elevated M_sum during periods of increased flight costs such as migration (Swanson, 1995; Swanson and Dean, 1999), the cold acclimatization and flight hypotheses. The cold acclimatization hypothesis contends that migrants encounter colder temperatures while moving to higher latitudes during spring migration and that M_sum is elevated to support thermogenesis in these colder temperatures. The flight adaptation hypothesis proposes that adjustments promoting endurance flight during migration produce elevated M_sum as a by-product. Documentation of cross-training effects in this
study is consistent with the flight adaptation hypothesis. Cold-trained birds had a higher $M_{\text{sum}}/\text{MMR}$ ratio (67.7%) compared to exercise-trained birds (58.9%). Furthermore, cold training increased $M_{\text{sum}}$ and MMR by 14.6% and 12.1% respectively, whereas exercise training increased $M_{\text{sum}}$ and MMR by 15.5% and 19.7%. Thus, cold training affected thermogenic capacity more than exercise capacity, whereas the opposite pattern occurred for exercise training. This result suggests that while cross-training effects occur, specific training enhances the capacity for the muscular activity involved in the training to a greater degree than capacities for other types of muscular activity.

To our knowledge, this study is the first to document that cold exposure can increase exercise capacity in birds. Results from studies of cross-training effects in mammals are difficult to generalize, with some studies documenting cross-training effects (Hayes and Chappell, 1986; McDonald et al., 1988; Turner et al., 1995; Schaeffer et al., 2001; Boström et al., 2012) and other studies failing to document such effects (Conley et al., 1985; Schaefer et al., 1996). One potential reason for this inconsistency is because exercise and thermogenesis are not completely functions of the same tissues in small mammals, as brown fat contributes substantially to thermogenesis but not to exercise in many cold-acclimated small mammals (Thompson et al., 2012). For example, cold acclimation in rodents produces hypertrophy and elevated metabolism in brown adipose tissue (Smith and Roberts, 1964; Cannon and Nedergaard, 2004). In birds, which lack brown adipose tissue (Mezentseva et al., 2008), both exercise and thermogenesis are functions of skeletal muscle activity, so similar adjustments to exercise and cold exposure seem more likely for birds than for mammals.

Cold and exercise training produced different trends in BMR for sparrows in this study, with a significant decrement of BMR for exercise-trained birds and a non-significant trend toward higher BMR in cold-trained birds, even though both training protocols increased maximum metabolic outputs. Studies are still too limited to form firm conclusions about training effects on BMR in birds, but studies to date on exercise training and BMR have documented significant decreases in BMR for exercise-trained relative to control birds (Deerenberg et al., 1998; Nudds and Bryant, 2001). Our results agree with these previous studies and, therefore, are consistent with the compensation hypothesis of Deerenberg et al. (1998), which states that reduced BMR compensates for high exercise-induced metabolic rates, thereby allowing a relatively lower daily energy expenditure (DEE). We did not measure DEE in this study, so we cannot confirm that similar compensation occurred in our exercise-trained birds, but the reduced BMR in this group is at least consistent with such a scenario.
It is important to point out, however, that the reduced BMR from both previous studies was at least partly due to reduced \( M_b \) in trained birds. In contrast, the exercise-trained group in our study exhibited lower BMR, even with an increase of \( M_b \) and pectoral muscle masses (see below). Reductions in overall body mass for previous studies were at least partly due to altered fat rather than lean mass (Nudds and Bryant, 2001). Migratory birds also often decrease BMR after migration, but this is primarily a function of decreased lean mass (Battley et al., 2001). In our study, fat scores for exercise-trained and control groups were not significantly different, so increases in lean mass, including pectoralis muscle mass, were major components of the elevated \( M_b \). A similar reduction in BMR after training, despite stable or increasing \( M_b \), has also been observed in mammals (Westerterp et al., 1994; Hoppeler et al., 1995).

On the contrary, winter BMR is usually higher than in summer for wild birds in seasonal climates, but it may also be lower or seasonally stable (Dawson and O’Connor, 1996; McKechnie, 2008), although some of this variation is likely explained by differences in climate (van de Ven et al., 2013a). Cold acclimated birds generally show 5 to 42% increases in BMR compared to their warm acclimated counterparts (Klaassen et al., 2004; Vézina et al., 2006; McKechnie, 2008; Peña-Villalobos et al., 2014). The increase in BMR produced by our acute cold training in the present study (15%) is at the lower end of this range, perhaps due to differences in daily energy expenditure (DEE) related to prolonged vs. acute cold exposure. However, we didn’t measure DEE in this study, so the question of whether acute severe cold training produces differences in DEE compared to prolonged, less severe cold acclimation protocols and whether such differences impact BMR will require further study. The impact of acute cold training on BMR has not previously been investigated in birds, but in mammals, short daily cold exposures induced higher BMR, similar to cold acclimation (Heldmaier et al., 1989; Wiesinger et al., 1990), so our results for house sparrows are consistent with these mammalian studies.

The aerobic capacity model for the evolution of endothermy posits a positive correlation between BMR and maximum metabolic rates in birds and mammals (Bennett and Ruben, 1979). Bennett and Ruben (1979) proposed that BMR is subject to two conflicting selective forces, increased sustained activity, which results in elevations of aerobic capacity and BMR, and maximized energy conservation, which selects for reduced BMR. In the present study, cold training increased BMR associated with increased aerobic capacity, whereas exercise training modified BMR towards energy conservation, despite increases in maximal metabolic outputs. Thus, BMR and maximal metabolic outputs did not consistently vary in the same directions with cold and exercise training. Moreover, neither whole-organism nor mass-independent BMR were
significantly correlated with whole-organism or mass-independent $M_{\text{sum}}$ or MMR, either within training groups or for combined data for all sparrows. Results of intraspecific tests of the aerobic capacity model assumption of links between BMR and maximal metabolic outputs are inconsistent (Hayes and Garland, 1995; Boily, 2002; Vézina et al., 2006; Lewden et al., 2012; Swanson et al., 2012; Petit et al., 2013), and our results in this study did not consistently support the assumption of a phenotypic link between minimum and maximum metabolic outputs. We did, however, observe a significant positive correlation between both raw and mass-independent metabolic rates for $M_{\text{sum}}$ and MMR, which contrasts with previous studies (Wiersma et al., 2007; Swanson et al., 2012) and suggests that adjustments in physiology for exercise and shivering occur via similar mechanistic bases.

$M_b$ increased between pre- and post-treatment measurements for both training groups, whereas $M_b$ stayed fairly constant for both control groups (Table 1). Post-training $M_b$ was also higher for both training groups relative to their control groups. These data indicate a consistent effect of both cold and exercise training in promoting increments of $M_b$ in house sparrows. $M_b$ generally increases during both migratory (Piersma et al., 1995; Aamidor et al., 2011) and wintering (Swanson, 1991; Liknes and Swanson, 2011) seasons for small birds. Cold acclimation may also increase $M_b$ for some species (Vézina et al., 2006; Vézina et al., 2011; Swanson et al., 2014b). However, other previous exercise-training and cold-acclimation studies showed stable (Klaassen et al., 2004; Bauchinger et al., 2010) or reduced (Deerenberg et al., 1998; Swaddle and Biewener, 2000; Nudds and Bryant, 2001) $M_b$ compared to control groups for other bird species. Our sparrows were provided more diet options, such as a protein supplement and meal worms, compared to studies documenting stable $M_b$ under cold or exercise protocols, so differences in diets among studies might contribute to the different results. We did not measure food intake in the current study, but increments of $M_b$ for both training groups suggested that overall food intake increases for birds on both of our training protocols, providing the necessary energy to modify phenotypes.

Both training protocols increased pectoralis mass for house sparrows in the present study. In contrast, several studies of cold acclimation in captive birds documented stable pectoralis mass between cold acclimated and control birds (Vézina et al., 2006; Peña-Villalobos et al., 2014; Swanson et al., 2014b). However, winter birds in cold climates generally show increases in pectoralis mass relative to summer, and these increases range from 5-33% (Swanson and Vézina, 2015), encompassing the 7% increase with cold training that we documented for sparrows in this study. Thus, acute cold training appears to produce pectoralis hypertrophy of a similar magnitude to that for winter acclimatization for birds wintering in cold climates. Exercise training, on the
other hand, may reduce pectoralis mass to decrease wing loading and force-generating capacity associated with high-energy take-off flights (Swaddle and Biewener, 2000). In this study, BMR was lower for exercise-trained than for control birds, which is consistent with such an energy conservation strategy, despite the increase in pectoralis mass and higher maximum metabolic outputs for exercise-trained birds. Feather clipping to increase flight costs in birds also produces increases in pectoralis mass (Lind and Jakobsson, 2001; Petit and Vézina, 2014) and migratory birds also consistently show pectoralis muscle hypertrophy during migration to help support prolonged flights (reviewed in Swanson, 2010; Piersma and van Gils, 2010). The exercise-induced increase in pectoralis muscle mass in this study is thus consistent with these latter adjustments to increase power production for extended flights.

Cold training did not affect heart mass in sparrows, whereas in cold acclimated and winter acclimatized birds, heart mass often increases (Liknes and Swanson, 2011; Peña-Villalobos et al., 2014; Swanson et al., 2014a; Swanson et al., 2014b). In contrast, exercise training increased heart mass for sparrows in this study. Migration also typically results in increments of heart mass (Dawson et al., 1983; Piersma, 1998; Vézina et al., 2007), so exercise training produced similar adjustments in heart mass as in migrant birds, which suggests that heart mass increases are a common contributor to enhanced endurance exercise. Why patterns of heart mass variation differed between cold and exercise training in this study is unknown, but the differing patterns might be related to the lower maximal metabolic outputs generated for cold training than for exercise training in this study.

Reductions in myostatin protein expression occurred for both training groups, which is consistent with a role for myostatin in mediating training-induced increases in pectoralis muscle mass. Neither training protocol, however, induced significant variation of myostatin mRNA expression, despite myostatin mRNA expression in sparrows being significantly positively correlated with protein levels of the latent form of myostatin. Such a correlation was not detected in a study of winter phenotypic flexibility of black-capped chickadees and American goldfinches, *Spinus tristis* (Swanson et al., 2014a). mRNA expression for both TLLs was higher in trained birds, significantly so for cold-trained birds, suggesting that myostatin processing capacity actually increased in cold- and exercised-trained birds. This result is contrary to our hypothesis of reduced TLL expression and, therefore, reduced myostatin processing capacity. The TLL metalloproteinases, however, have many other functions in birds and mammals, including roles in immune function, cleavage of chordin during bone formation and activation of lysyl oxidase for formation of covalent cross-links in collagen and elastic fibers during exercise (Clark et al., 1999; Scott et al., 1999). Moreover, TLLs and tolloid like receptors may be generally upregulated by
exercise (Lancaster et al., 2005). Higher TLL mRNA expression for both training groups did not result in increased ratios of active-to-latent myostatin protein ($P > 0.64$ for both groups), as protein levels of both latent and active forms of myostatin decreased for cold- and exercise-trained sparrows relative to controls. The present study suggests that post-transcriptional regulation of myostatin is an important regulatory mechanism for metabolic flexibility for both cold and exercise training. The reduced pectoralis myostatin protein levels with increased energy demands for both training groups is generally consistent with trends in myostatin expression during winter acclimatization in small birds, including house sparrows (Swanson et al., 2009; Swanson et al., 2014a). However, reduced pectoralis myostatin mRNA and TLL expression has not been previously documented during migration, although pectoralis myostatin protein levels may decrease in the migratory state (Price et al., 2011; King et al., 2015).

In summary, this is the first direct demonstration of cross-training effects of cold on MMR and exercise on $M_{\text{sum}}$ in birds. The occurrence of cross-training effects agrees with previous data documenting effects of feather clipping (and increasing flight costs) and migration on $M_{\text{sum}}$ (Swanson and Dean, 1999; Vézina et al., 2007; Petit and Vézina, 2014; Corder and Schaeffer, 2015). Cold and exercise training-induced phenotypic flexibility in the current study is consistent with that documented for migration and winter acclimatization (Swanson, 2010; Swanson et al., 2014a), suggesting clear ecological connections and potential fitness consequences for flexible phenotypes. However, other studies suggest that metabolic flexibility is not only due to adjustments in lean mass (e.g., muscles), but also results from alterations of the metabolic characteristics of the lean tissue (Florez-Duquet and McDonald, 1998). Further studies are needed to address the question of whether changes in cellular metabolic intensity and lipid transport capacity also contribute to cross-training effects, as they do for migratory and winter phenotypes (Guglielmo, 2010; Swanson, 2010).

**Materials and Methods**

All procedures in this study were approved by the University of South Dakota Institutional Animal Care and Use Committee (Protocol 79-01-11-14C).

**Bird Capture**

We captured house sparrows by mist net from wild populations near Vermillion, Clay County, South Dakota (approximately 42° 47’ N, 97° W) during September (Cold-training experiments, $n = 18$) of 2012 and March (Exercise-training experiments, $n = 16$) of 2013. After
capture, we transported birds back to the University of South Dakota Department of Biology Animal Facility, where sparrows were weighed and housed individually in 59 cm × 45 cm × 36 cm stainless-steel cages at room temperature (23°C ± 2°C) and 12 L:12 D photoperiod. We acclimated birds to captive conditions with ad libitum mixed seed, protein supplement (mixture of homogenized dog food and hard-boiled egg), six mealworms per day, and water with added vitamins (Wild Harvest Multi-Drops vitamin supplement for all birds, United Pet Group, Inc., Cincinnati, OH) for two weeks before we measured pre-training metabolic rates.

Respirometry

We measured metabolic rates using open-circuit respirometry as described in Swanson et al. (2012). Briefly, we followed a standardized sequence for metabolic tests, with BMR measured first at night, then MMR the next morning between 9:00 am and noon, followed by a rest period of at least four hours before \( M_{\text{sum}} \) measurement. Between metabolic measurements, sparrows were returned to their cages and provided free access to food and water. Body masses were measured before and after each metabolic rate measurement. The respirometry system for BMR and \( M_{\text{sum}} \) measurements consisted of 1.8-L paint cans with the inner surface painted flat black as metabolic chambers. Metabolic chambers were immersed into an ethylene glycol bath for temperature control to ± 0.2°C. We maintained flow rates of dry, CO\(_2\)-free helox (79% helium/21% oxygen) at 1,010–1,030 ml min\(^{-1}\) for \( M_{\text{sum}} \) measurements. For BMR and MMR measurements, we used dry, CO\(_2\)-free air instead of helox as the respiratory gas and maintained flow rates at 280–300 ml min\(^{-1}\) for BMR and 1,730–1,760 ml min\(^{-1}\) for MMR. We controlled flow rates with a Cole-Parmer Precision Rotameter (Model FM082–03ST) calibrated to ± 1% accuracy with a soap bubble meter. We sampled fractional concentrations of oxygen in excurrent air with an Ametek S-3A oxygen analyzer (Applied Electrochemistry, Pittsburgh, PA, USA) at 1 sec intervals and collected data with Expedata 2.0 (Sable Systems, Henderson, NV, USA) software. We also analyzed oxygen consumption data with Expedata 2.0 software after correcting to STPD, using steady state calculations for BMR and instantaneous calculations for \( M_{\text{sum}} \) and MMR (Bartholomew et al., 1981).

Metabolic Measurements

We conducted BMR measurements at night (at least one hour after darkness) on birds fasted for at least four hours prior to metabolic measurements at 30°C, which is within the thermoneutral zone of house sparrows (Arens and Cooper, 2005). We allowed birds an
equilibration period of at least 1 h within the metabolic chamber before we initiated metabolic measurements. We measured BMR for all birds for a period of at least 3 hours following the 1-h equilibration periods and we calculated 10-min running mean values for oxygen consumption over the test period, with the lowest 10-min running mean designated as BMR.

We used a rotating hop-flutter wheel to generate exercise-induced MMR (Chappell et al., 1999; Wiersma et al., 2007; Swanson et al., 2012). Our hop-flutter chamber was designed from a 30-cm diameter × 14-cm width piece of PVC pipe affixed with air-tight acrylic side panels. We attached the chamber to a variable speed motor to control rotation speed and placed five ping-pong balls in the chamber to help motivate the bird to exercise as the chamber was turning (Swanson et al., 2012). Prior to MMR measurements, we allowed a 5-min equilibration period, during which the chamber was covered by a sheet to calm the bird, before we initiated chamber rotation. After removing the sheet, we initiated chamber rotation at the lowest speed on the motor for 3 min and increased the rotation speed every 3 min thereafter until the oxygen consumption plateaued and the bird showed reluctance to exercise. After termination of the rotation, birds were retained inside the chamber for at least 5 min until oxygen consumption decreased. All birds invariably showed signs of fatigue at the end of MMR tests (e.g., resting their breast on the chamber floor and panting heavily), suggesting that maximum aerobic activity during the hop-flutter exercise had been attained.

We elicited $M_{\text{sum}}$ with a sliding cold exposure protocol with helox (Swanson et al., 1996), which increases heat loss relative to air (Arens and Cooper, 2005). For the sliding cold exposure protocol, after flushing the chamber with helox for 5 min, we initiated the cold exposure by immersing the metabolic chamber into the anti-freeze bath, with the cold exposure initiated at 0 to -5°C. We continued the sliding cold exposure treatment until we detected a steady decline in oxygen consumption over several minutes, which is indicative of hypothermia. We then removed birds from the metabolic chamber and recorded body temperatures cloacally. We considered body temperatures of $\leq 36^\circ\text{C}$ as hypothermic (Swanson and Liknes, 2006) and all birds were hypothermic at the end of cold exposure trials, which validated that $M_{\text{sum}}$ had been attained.

Training Protocols

After pre-training metabolic rate measurements, we randomly assigned sparrows to either training (cold or exercise) or control experimental groups. Both training and control groups followed the same schedule, which included six three-day training sessions with one day of rest in between sessions (24 days total). Acute cold training ($n=9$) was performed in helox by placing birds inside the metabolic chamber for 45 min at 10°C for the first training session, with 2°C
reductions for each successive 3-d session, with the last session at 0°C. Cold-training controls (n=9) were placed inside the metabolic chamber in air at 30°C for 45 min during training days.

Exercise training (n=8) procedures were modified from Bauchinger et al. (2010). We trained sparrows to fly non-stop for 45 min between two perches located 6 m apart in a flight arena. During the first three-day training session, a person continuously walked after birds between the two perches with a training signal (hand clapping) when birds landed on perches. After the first three-day training session, all sparrows flew consistently between perches with only a training signal (hand clapping) and shortly thereafter, birds flew between perches continuously whenever a person was present in the room. Exercise-trained birds flew an average of approximately 4900 m per day during training bouts. For the exercise-training control group (n=8), sparrows were placed inside a cloth bag for 45 min, which generates a handling stress (e.g., Liu and Swanson, 2014a) without exercise.

Tissue Dissection

Following the last day of the training schedule, we measured post-training metabolic rates in the same order as pre-training measurements. The day following the final metabolic rate measurements, we euthanized birds by cervical dislocation. Fat stores of each bird were measured on a 0–5 scale following Liu and Swanson (2014b), and the pectoralis muscles and heart were then quickly excised on ice. After dissection, we weighed tissues to the nearest 0.1 mg before dividing samples into two aliquots, 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. Both samples were stored frozen at -80°C for later analyses.

qRT-PCR and Western Blot

We extracted total RNA from pectoralis samples using β-mercaptoethanol and the RNeasy Fibrous Tissue Mini kit (QIAGEN, Valencia, CA) and quantified total RNA using an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA). We used 50 ng of purified RNA for qRT-PCR reactions with a TaqMan RNA-to-C_T 1-Step Kit and StepOnePlus Real-Time PCR System (Applied Biosystems, Carlsbad, CA, USA). We performed qRT-PCR using 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 Swanson et al. (2009) (GenBank Accession Numbers KP337454-KP337456). We used glyceraldehyde phosphate dehydrogenase (GAPDH, Applied Biosystems) as a housekeeping
gene. For all qRT-PCR reactions, we used 6 μl of total RNA in 25 μl reactions. Each sample was run in duplicate and normalized to the expression of GAPDH. We performed 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 four genes to verify efficiency for these probe and primer sets for sparrows. Slopes and efficiencies for each gene were: GAPDH (-3.46, 94.6%), Myostatin (-3.52, 92.4%), TLL-1 (-3.34, 99.4%), TLL-2 (-3.42, 95.9%), respectively. We quantified changes in mRNA expression using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001; Arendt et al., 2012), comparing all samples to a reference sample. We used the mean value for all seasons for each tissue 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 for all other samples for the same tissue and species. We present mRNA expression data as relative expression levels (i.e., mean fold change ± SE).

We conducted Western blots on pectoralis muscles to analyze myostatin protein levels using antibodies raised against myostatin and GAPDH (as a housekeeping protein). We homogenized muscles on ice in a homogenizing buffer containing 50 mM Tris, pH 7; 100 mM NaCl; 2% SDS. We used a Cole-Parmer (Chicago, IL, USA) 4710 Series Ultrasonic homogenizer for homogenization, using three 10 sec bursts with 30 sec between bursts. Protein concentrations were determined using a modified DC Lowry improved protein assay and we used 20 μg of protein for analysis via sodium dodecyl sulfate-polyacrylamide gel electrophoresis. All samples were run 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. We conducted Western blotting using primary antibodies for myostatin (goat polyclonal; R&D Systems, Minneapolis, MN; 1:100 dilution) and GAPDH (chicken polyclonal; Millipore, Temecula, CA; 1:8,000 dilution). We incubated membranes in TBS-T with 5% milk (20mmol Tris, 137mM NaCl, 100mM HCl, 0.01% Tween 20, pH 7.5) overnight at 4°C. We next incubated membranes for 1 h at room temperature with the primary antibodies for myostatin and GAPDH, washed membranes three times for 5 min each with TBS-T, and incubated them with secondary antibodies for myostatin (anti-Goat; 1:1000; Santa Cruz Biotechnology, Dallas, TX, USA) and GAPDH (anti-chicken; 1:1,500; Abcam, Cambridge, MA, USA) conjugated to horseradish peroxidase for 1 h at room temperature. The protein samples were visualized using enhanced chemiluminescence (GE Healthcare ECL Plus Western Blotting Detection Reagents; Buckinghamshire, UK) and analyzed using Quantity One software (Bio-Rad Laboratories, Hercules, CA, USA).
Statistics

We present data as means ± standard error (SE), unless otherwise stated. All data were log_{10}-transformed prior to analyses and we tested for outliers with Grubbs’ test (Grubbs, 1950), removing outliers before subsequent statistical comparisons. We conducted least squares linear regression of log_{10}-transformed metabolic rates against log_{10}-transformed M_b for each training group and control for both pre- and post-treatment measurements and compared regression lines by ANCOVA after confirming statistically homogenous slopes (all $P > 0.240$ for comparisons of slopes). Linear regressions of log_{10}-transformed M_b vs. log_{10}-transformed pectoralis and heart masses were also compared between training and control groups by ANCOVA after verifying statistically homogenous slopes (all $P > 0.125$ for comparisons of slopes). For these regressions, we used $M_b -$ organ mass (both sides combined if muscles are paired) for the $M_b$ term to avoid statistical problems associated with part-whole correlations. We used SAS software (Version 9.3, SAS Institute Inc., Cary, NC, USA) for least squares regressions and ANCOVA. We compared mRNA and protein expression between training and control groups by Student’s $t$-test with SigmaStat 3.5 (Systat Software, Inc., Point Richmond, CA, USA). We further conducted least-squares linear regression and multiple regression with $M_b$ as a covariate (i.e., mass-independent metabolic rates) in SAS to examine correlations among metabolic rates, mRNA expression and protein levels of myostatin. We accepted statistical significance for all tests at $P < 0.05$.

Acknowledgments

We thank Donis Drappeau, Jake Johnson, Travis Carter and Bob Garner for technical support in the laboratory and field. We thank Ken Renner, Sol Redlin and Kathie Rasmussen for access to their property for collection of house sparrows. We also thank Jianqiu Zou and Yi-Fan Li for their advice on Western blots. We also thank Mark Dixon for his help on statistics. Two anonymous reviewers provided constructive comments on a previous version of this manuscript, and we thank them for their efforts. This research was funded by NSF IOS-1021218 to DLS. Research reported in this manuscript was also supported by an Institutional Development Award (IDeA) from the National Institute of General Medical Sciences of the National Institutes of Health under grant number P20GM103443. Its contents are solely the responsibility of the authors and do not necessarily represent official views of NIGMS or NIH.
List of symbols/abbreviations

BMR: basal metabolic rate
GAPDH: Glyceraldehyde 3-phosphate dehydrogenase
M_b: body mass
MMR: maximum metabolic rate (maximum exercise metabolic rate)
MSTN: myostatin
M_sum: summit metabolic rate (maximum thermogenic metabolic rate)
PEC: pectoralis
TGF-β: Transforming growth factor beta
TLL-1: tolloid-like metalloproteinase -1
TLL-2: tolloid-like metalloproteinase -2

Funding

This study was supported by the US National Science Foundation [grant number IOS-1021218 to D.S.]. Research reported in this manuscript was also supported by an Institutional Development Award (IDeA) from the National Institute of General Medical Sciences of the National Institutes of Health under grant number P20GM103443. Its contents are solely the responsibility of the authors and do not necessarily represent official views of NIGMS or NIH.

Author contributions

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

Competing interests

The authors declare no competing financial interests.
References


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Table 1. Mean ± SE body masses prior to BMR measurement for house sparrows before (pre-training) and after (post-training) 3-week cold and exercise training protocols compared to their controls. The values for “Change” represent post-treatment body mass minus pre-treatment body mass. * $P < 0.05$

<table>
<thead>
<tr>
<th></th>
<th>Cold training</th>
<th>Cold training control</th>
<th>Exercise training</th>
<th>Exercise training control</th>
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<tbody>
<tr>
<td>Post-training</td>
<td>28.622 ± 2.468*</td>
<td>26.444 ± 1.553</td>
<td>27.887 ± 1.709*</td>
<td>26.013 ± 0.976</td>
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<tr>
<td>Change (Post – Pre)</td>
<td>1.889 ± 1.641*</td>
<td>0.133 ± 1.014</td>
<td>1.700 ± 1.054*</td>
<td>-0.487 ± 1.068</td>
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Figure 1. Least squares linear regressions of log_{10}-transformed metabolic rates against log_{10} body mass (M_b) for cold-trained (filled circles) and control (open circles) house sparrows. Bar graphs on the right show least squares means (± SE) for each metabolic rate for cold-trained and control sparrows. Slopes did not differ significantly for all regressions. * P < 0.05.
Figure 2. Least squares linear regressions of log_{10}-transformed metabolic rates against log_{10} body mass (M_b) for exercise-trained (filled circles) and control (open circles) house sparrows. Bar graphs on the right show least squares means (± SE) for each metabolic rate for exercise-trained and control sparrows. Slopes did not differ significantly for all regressions. * P < 0.05.
Figure 3. **Linear least squares regressions of log\textsubscript{10}-transformed pectoralis (PEC; right side only) and heart masses on log\textsubscript{10} body mass (M\textsubscript{b}) for trained (filled circles) and control (open circles) house sparrows.** Body mass values for these regressions were M\textsubscript{b} minus the mass of the organ in question. Bar graphs below show least squares means (± SE) for each tissue mass for trained and control birds. Slopes did not differ significantly for all regressions. * \( P < 0.05.\)
Figure 4. Top Panels: Relative mRNA expression from qRT-PCR for *myostatin* and its metalloproteinase activators *TLL-1* and *TLL-2* in pectoralis for cold- and exercise-trained sparrows relative to control birds. Bottom Panels: Relative protein levels from Western blot for both unprocessed latent and active forms of *myostatin* (MSTN) for cold- and exercise-trained sparrows relative to control birds. *P < 0.05.*