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RESEARCH ARTICLE

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_{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 experimental cold and exercise training protocols for 3 weeks on body (M_b) and muscle (M_{pec}) masses, basal metabolic rate (BMR), M_{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_{sum}, MMR, M_b and M_{pec}, but BMR increased with cold training and decreased with exercise training. No significant differences occurred for pectoralis myostatin mRNA expression, but cold and exercise increased the expression of TLL-1 and TLL-2. Pectoralis myostatin protein levels were generally reduced for both training groups. These data clearly demonstrate cross-training effects of cold and exercise in birds, and are consistent with a role for myostatin in increasing pectoralis muscle mass and driving organismal increases in metabolic capacities.

KEY WORDS: Phenotypic flexibility, Cold training, Exercise training, Pectoralis, Myostatin, MMR, M_{sum}, BMR, Birds

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 (M_{pec}), 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, 2014b; Petit and Vézina, 2014). 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 that 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 TLL proteins in birds during periods (migration and winter acclimatization) that cause variation in muscle mass (Swanson et al., 2009, 2014a; Price et al., 2011; 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

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List of symbols and abbreviations

BMR	basal metabolic rate
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
M_b	body mass
M_{heart}	heart mass
M_{pec}	pectoralis muscle 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

expression of myostatin and the TLLs and M_{pec} (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_{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_{sum} and MMR because of the lack of 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_{sum} and M_{pec} along with winter decreases in expression of mRNA encoding myostatin and TLL-1 (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_{sum} and MMR before and after training treatments. We also measured skeletal muscle and heart masses, pectoralis expression of mRNA encoding myostatin and TLL proteins 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) (Fig. 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_{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 (Fig. 2). After training, exercise-trained birds also showed significantly elevated M_{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_{sum} (11.6%, $F_{1,16}$=4.77, P=0.047) and MMR (24.4%, $F_{1,13}$=31.75, P<0.001) compared with cold-trained birds. Between post-training control groups, exercise-training control birds had significantly higher M_{sum} (12.9%, $F_{1,15}$=5.97, P=0.029) and MMR (18.7%, $F_{1,14}$=42.78, P<0.001) compared with 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_{sum}/MMR ratios (0.68±0.09) compared with exercise-trained birds (0.59±0.07), whereas no differences in M_{sum}/MMR ratios occurred between cold-trained (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-trained (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 (Fig. 3). Exercise-trained birds showed non-significant trends toward increases in both M_{pec} ($F_{1,15}$=4.13, P=0.063) and M_{heart} ($F_{1,15}$=3.64, P=0.079) relative to control birds (Fig. 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

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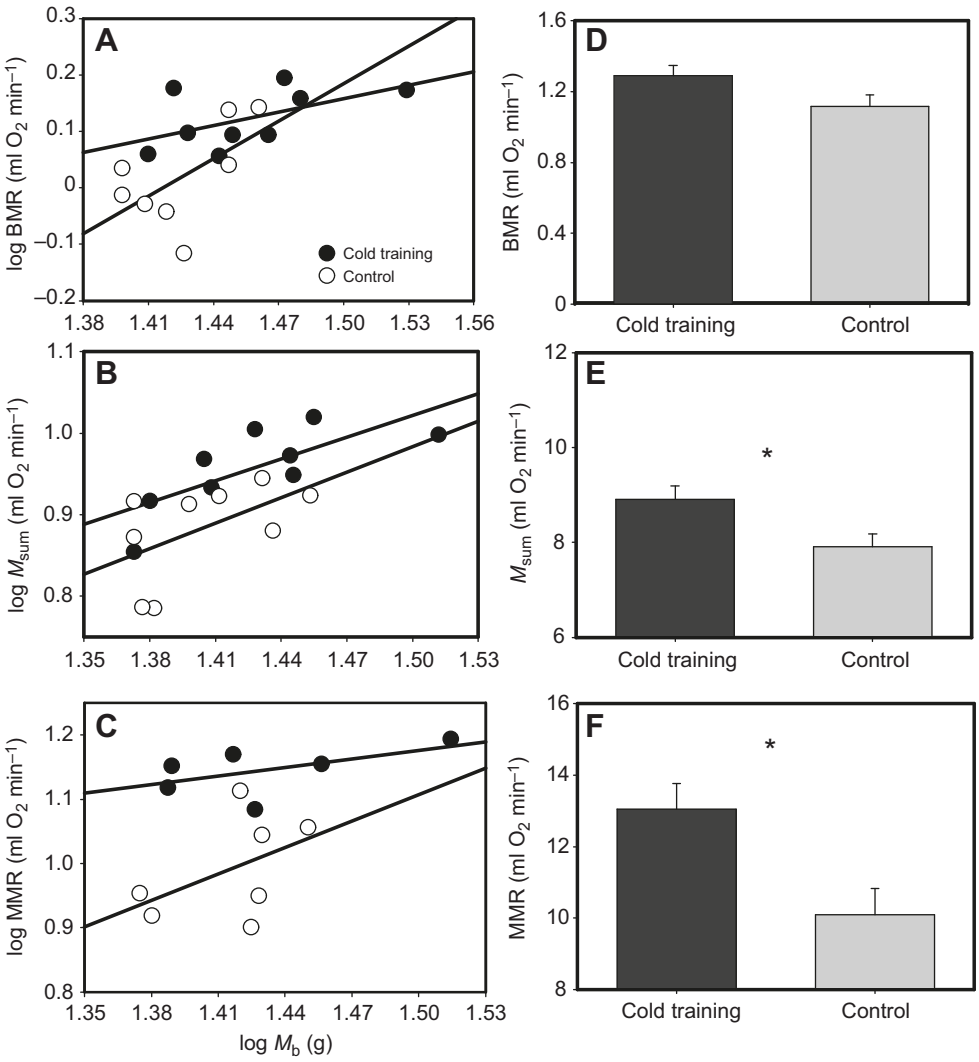

Fig. 1. BMR, M_{sum} and MMR in cold-trained house sparrows. (A–C) Least-squares linear regressions of $\log_{10}$-transformed metabolic rates against $\log_{10}$ body mass (M_b) for cold-trained and control house sparrows. Slopes did not differ significantly for all regressions. (D–F) Least-squares means (±s.e.) for each metabolic rate for cold-trained and control sparrows. *P<0.05.

average expression in control compared with cold-trained groups (Fig. 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 birds 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 birds compared with 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 (Fig. 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 (Fig. 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_{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_{sum} and MMR. Latent myostatin protein levels were significantly negatively correlated with mass-independent M_{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 the thermogenic capacity of sparrows, similar to cold acclimation protocols in other bird studies (McKechnie, 2008; Swanson, 2010). M_{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 the

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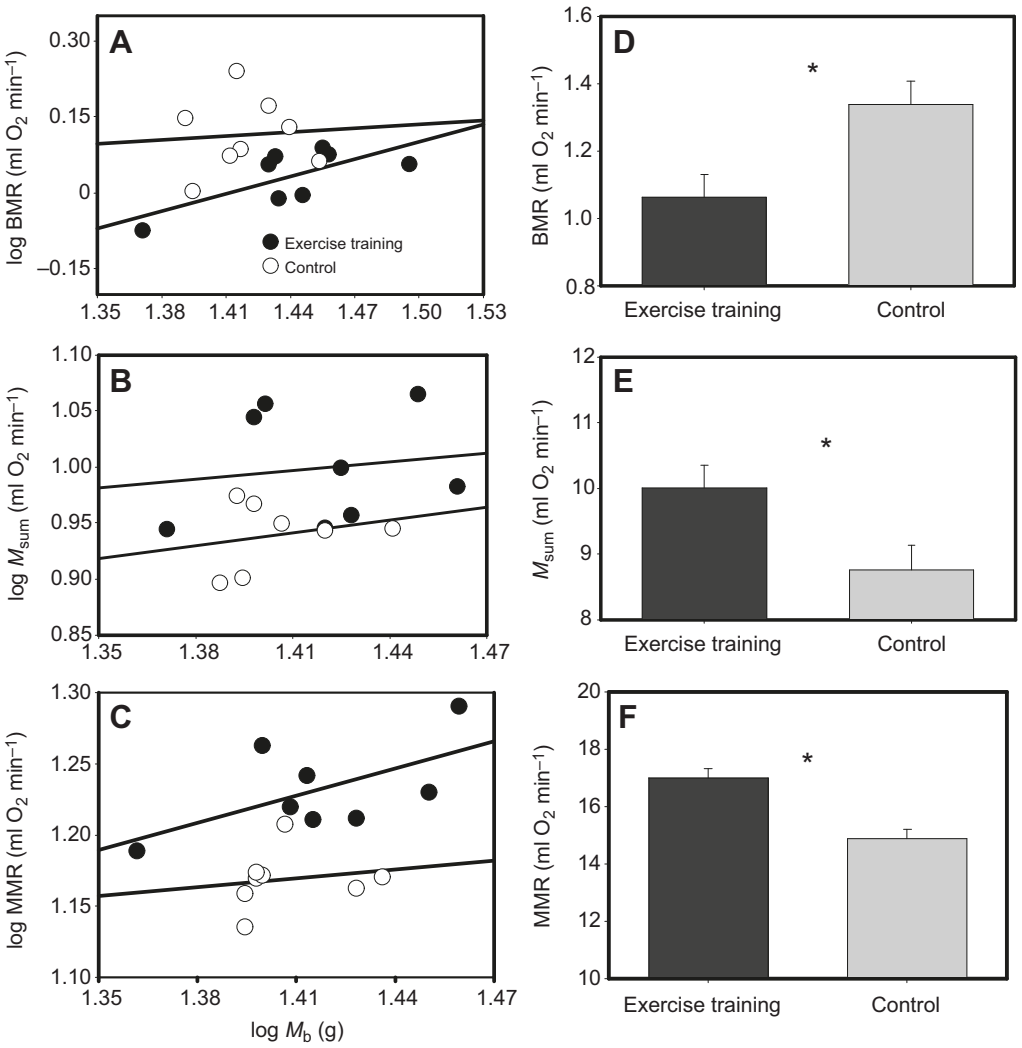

Fig. 2. BMR, M_{sum} and MMR in exercise-trained house sparrows. (A–C) Least-squares linear regressions of $\log_{10}$-transformed metabolic rates against $\log_{10}$ body mass (M_b) for exercise-trained and control house sparrows. Slopes did not differ significantly for all regressions. (D–F) Least-squares means (±s.e.) for each metabolic rate for exercise-trained and control sparrows. *P<0.05.

percentage 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 with 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).

Effects of exercise and cold cross-training 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 could also 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

Table 1. Body masses prior to BMR measurement for house sparrows before and after cold and exercise training protocols

	M_b (g)			
	Cold training	Cold-training control	Exercise training	Exercise-training control
Pre-training	26.733±1.726	26.311±1.330	26.188±2.090	26.500±1.350
Post-training	28.622±2.468*	26.444±1.553	27.887±1.709*	26.013±0.976
Change (Post–Pre)	1.889±1.641*	0.133±1.014	1.700±1.054*	–0.487±1.068

The values for 'change' represent post-treatment body mass minus pre-treatment body mass. *P<0.05.

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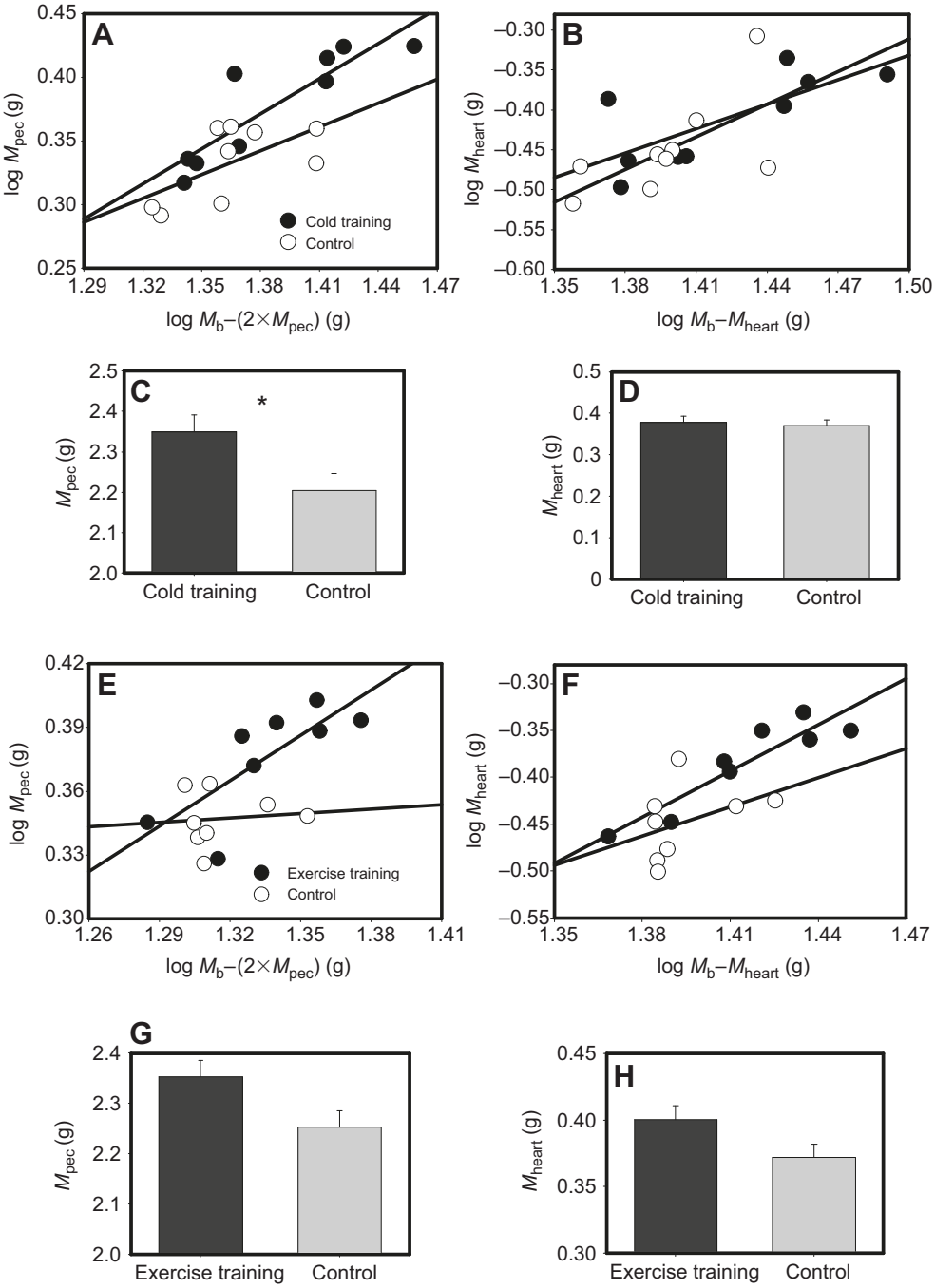

Fig. 3. Mass of pectoralis and heart muscle in cold-trained and exercise-trained house sparrows. Linear least-squares regressions of $\log_{10}$-transformed pectoralis (M_{pec}; right side only) and heart (M_{heart}) masses on $\log_{10}$ body mass (M_b) for cold-trained (A,B) and exercise-trained (E,F) house sparrows. Body mass values for these regressions were M_b minus the mass of the organ in question. Slopes did not differ significantly for all regressions. Bar graphs show least squares means (±s.e.) in trained and control birds for M_{pec} (C,G) and M_{heart} (D,H). *P<0.05.

increase. Conversely, elevated physiological adjustments producing a 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 phenomemon 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_{sum}/MMR ratio (67.7%) compared with exercise-trained birds (58.9%). Furthermore, cold training increased M_{sum} and MMR by 14.6% and 12.1%, respectively, whereas exercise training increased M_{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 although cross-training effects occur, specific training enhances the

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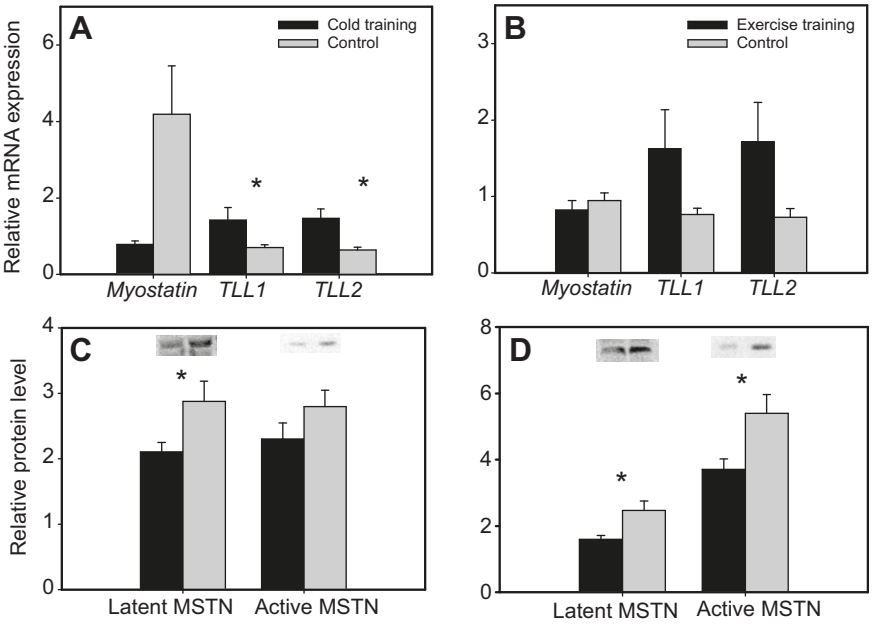

Fig. 4. Protein and mRNA expression of myostatin, TLL-1 and TLL-2 in cold-trained and exercise-trained house sparrows. Relative mRNA expression from qRT-PCR for *Myostatin* and its metalloproteinase activators *TLL1* and *TLL2* in pectoralis for cold- (A) and exercise-trained (B) sparrows relative to control birds. Relative protein levels from western blot for both unprocessed latent and active forms of myostatin (MSTN) for cold- (C) and exercise-trained (D) sparrows relative to control birds. **P*<0.05.

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 that exercise and thermogensis are not functions of the same tissues in small mammals, because 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 mass (M_{pec}; 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 M_{pec}, 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).

In contrast, winter BMR is usually higher than that 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 5–42% increases in BMR compared with 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 versus acute cold exposure. However, we did not measure DEE in this study, so the question of whether acute severe cold training produces differences in DEE compared with 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: (1) increased sustained activity, which results in elevations of aerobic capacity and BMR, and (2) maximized energy

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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_{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_{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, 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 with control groups for other bird species. Our sparrows were provided more diet options, such as a protein supplement and meal worms, compared with 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 M_{pec} for house sparrows in the present study. In contrast, several studies of cold acclimation in captive birds documented stable M_{pec} 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 M_{pec} relative to summer, and these increases range from 5 to 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, by contrast, may reduce M_{pec} 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 M_{pec} and higher maximum metabolic outputs for exercise-trained birds. Feather clipping to increase flight costs in birds also produces increases in M_{pec} (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 M_{pec} in this study is thus consistent with these latter adjustments to increase power production for extended flights.

Cold training did not affect M_{heart} in sparrows, whereas in cold-acclimated and winter-acclimatized birds, M_{heart} often increases (Liknes and Swanson, 2011; Peña-Villalobos et al., 2014; Swanson et al., 2014a,b). In contrast, exercise training increased M_{heart} for sparrows in this study. Migration also typically results in increments of M_{heart} (Dawson et al., 1983; Piersma, 1998; Vézina et al., 2007), so exercise training produced similar adjustments in M_{heart} as in migrant birds, which suggests that heart mass increases are a common contributor to enhanced endurance exercise. Why patterns of variation in M_{heart} 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 M_{pec}. Neither training protocol, however, induced significant variation of myostatin mRNA expression, despite such 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 capacity for myostatin processing. 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 levels of 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, 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_{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_{sum} (Swanson and Dean, 1999; Vézina et al., 2007; Petit and Vézina, 2014; Corder and Schaeffer, 2015). Phenotypic flexibility induced by cold and exercise training in the

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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 (*Passer domesticus* Linnaeus 1758) by mist net from wild populations near Vermillion, Clay County, South Dakota (~42°47′N, 97°W) during September 2012 (cold-training experiments, N=18) and March 2013 (Exercise-training experiments, N=16). 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±2°C) and 12 h:12 h L: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 2 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 09:00 h and 12:00 h, followed by a rest period of at least 4 h before M_{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_{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 1010–1030 ml min^{-1} for M_{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 1730–1760 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 s 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_{sum} and MMR (Bartholomew et al., 1981).

Metabolic measurements

We conducted BMR measurements at night (at least 1 h after darkness) on birds fasted for at least 4 h 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 h 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 piece of PVC pipe 30 cm in diameter and 14 cm long, 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 encourage 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_{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 antifreeze 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 ≤36°C as hypothermic (Swanson and Liknes, 2006) and all birds were hypothermic at the end of cold exposure trials, which validated that M_{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 3 day training sessions with 1 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 day 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 3 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 3 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 day^{-1} 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 killed 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±s.e.).

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 mmol l^{-1} Tris-HCl, pH 7, 100 mmol l^{-1} NaCl, 2% SDS. We used a Cole-Parmer (Chicago, IL, USA) 4710 Series Ultrasonic homogenizer for homogenization, using three 10 s bursts with 30 s 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; 1:8000 dilution). We incubated membranes in TBS-T with 5% milk (20 mmol l^{-1} Tris, 137 mmol l^{-1} NaCl, 100 mmol l^{-1} 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; 1:1500; 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±s.e., 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 M_{pec} and M_{heart} 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 (v9.3, 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 $P<0.05$.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

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

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