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RESEARCH ARTICLE

Phenotype manipulations confirm the role of pectoral muscles and haematocrit in avian maximal thermogenic capacity

Magali Petit* and François Vézina

ABSTRACT

In small resident bird species living at northern latitudes, winter cold acclimatization is associated with an increase in pectoral muscle size and haematocrit level, and this is thought to drive the seasonal increase in summit metabolic rate ($\dot{M}_{sum}$, a measure of maximal shivering thermogenic capacity). However, evidence suggesting that pectoral muscle size influences $\dot{M}_{sum}$ is correlational and the link between haematrocrit level and $\dot{M}_{sum}$ remains to be demonstrated. We experimentally tested the relationship between pectoral muscle size and $\dot{M}_{sum}$ by manipulating muscle size using a feather clipping protocol in free-living wintering black-capped chickadees (*Poecile atricapillus*). This also allowed us to investigate the link between haematocrit and thermogenic capacity. After a first series of measures on all birds, we cut half of the flight feathers of experimental individuals (*N*=14) and compared their fat and pectoral muscle scores, $\dot{M}_{sum}$ and haematocrit level at recapture with their previous measures and with those of control birds (*N*=17) that were captured and recaptured at comparable times. Results showed that: (1) experimental birds developed larger pectoral muscles than control individuals and (2) mass-independent $\dot{M}_{sum}$ was up to 16% higher in birds expressing large pectoral muscles. $\dot{M}_{sum}$ was also positively correlated with haematocrit, which was not affected by the experimental manipulation. These findings demonstrate that, for a given body mass, large pectoral muscles are associated with a higher $\dot{M}_{sum}$ in black-capped chickadees and that oxygen carrying capacity likely supports thermogenesis in this species.

KEY WORDS: Summit metabolic rate, Shivering, Metabolic performance, Feather clipping, Haematocrit

INTRODUCTION

Birds overwintering at northern latitudes face low ambient temperatures and restricted food availability (McNamara et al., 1990; McNamara and Buchanan, 2005; Swanson, 2010). To survive in this environment, a suite of physiological traits must adjust to winter conditions (Swanson, 1991a; Cooper and Swanson, 1994; Swanson and Thomas, 2007). Specifically, winter acclimatization is associated with an increase in body mass (Carey et al., 1978; Williams and Tieleman, 2000; Vézina et al., 2006), fat reserves (Swanson, 1991a; Sharbaugh, 2001; Cooper, 2002), muscle mass (Swanson, 1991b; Cooper, 2002; Liknes and Swanson, 2011), summit metabolic rate ($\dot{M}_{sum}$) (Swanson, 1991a; Cooper and

Swanson, 1994; Vézina et al., 2006) and haematocrit (deGraw et al., 1979; Swanson, 1990b; O'Connor, 1996).
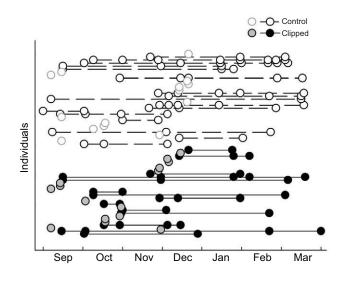
$\dot{M}_{sum}$ is a measure of maximal thermogenic capacity, and individuals with higher $\dot{M}_{sum}$ values have been shown to express better cold tolerance (Swanson, 1990a) and endurance (Swanson, 2001), meaning that they can face colder temperature over longer periods than birds with lower $\dot{M}_{sum}$ values. Because pectoral muscles are the largest muscles in birds (Marsh and Dawson, 1989; Swanson, 1991b; O'Connor, 1995), it is widely assumed that the increase in $\dot{M}_{sum}$ typically seen in cold-acclimatized birds (O'Connor, 1995; Cooper, 2002; Petit et al., 2013) results from the associated increase in pectoral muscle size (Cooper, 2002; Saarela and Hohtola, 2003; Vézina et al., 2007; Vézina et al., 2011), and recent findings by Swanson et al. (Swanson et al., 2013) in American goldfinches (*Spinus tristis*) support this interpretation. However, the link between $\dot{M}_{sum}$ and the size of pectoral muscles remains correlative and the relationship has yet to be tested experimentally. For instance, maximal heat production could also benefit from non-shivering heat sources such as elevated tissue metabolic intensity (Vézina et al., 2011; Zheng et al., 2013a; Zheng et al., 2013b), and cases of cold acclimation without changes in size of pectoral muscles have been reported (Williams and Tieleman, 2000; Vézina et al., 2006).

Haematocrit (the proportion of red blood cells in total blood) is a measure of maximal oxygen carrying capacity, which is thought to support overall metabolic activity (Carpenter, 1975; Burness et al., 1998; Hammond et al., 2000; Calbet et al., 2006), including thermogenesis (Carey and Morton, 1976; Swanson, 1990b). For instance, Swanson (Swanson, 1990b) found 11% higher haematocrit and 9% higher oxygen carrying capacity in dark-eyed juncos (*Junco hyemalis*) during winter relative to summer and showed in a parallel study that juncos also expressed elevated $\dot{M}_{sum}$ in winter (Swanson, 1990a). Winter increases in haematocrit are therefore interpreted as a physiological upregulation in response to elevated oxygen demands for thermogenesis (Carey and Morton, 1976; Swanson, 1990b), which likely maximizes heat production capacity and cold endurance. However, increasing the number of cells in circulation also increases blood viscosity, which suggests that the relationship between haematocrit and thermogenic capacity should not be linear but rather dome shaped, with an optimal haematocrit found at intermediate levels (Schuler et al., 2010), which may vary among seasons depending on heart size adjustments (e.g. Liknes and Swanson, 2011). The functional link between $\dot{M}_{sum}$ and haematocrit, however, remains to be demonstrated.

In this study, we manipulated the pectoral muscle size of free-living black-capped chickadees [*Poecile atricapillus* (Linnaeus 1766)] by cutting the primary and rectrix feathers (Sanz et al., 2000; Ardia and Clotfelter, 2007; Harding et al., 2009b) of experimental individuals (Fig. 1). This technique reduces wing and tail surface area, which has been shown to force birds to develop larger pectoral muscles to compensate for the loss in lift (Lind and Jakobsson, 2001). Based on the known correlation between pectoral muscle size

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Fig. 1. Captures and recaptures of black-capped chickadees. Each individual capture is represented by a circle. Grey circles represent individuals that were not recaptured. Circles joined by a line represent captures and recaptures for given individuals.

Fig. 2. Variation in body mass over the experimental period. Data are least square means of body mass for control and clipped black-capped chickadees before and after the treatment. Least square means were extracted from separated LME models per group controlling for period, body size and time of capture, with bird ID as random parameter. Asterisk indicates the significant increase in body mass within the control group between periods (***$P<0.001$).

and $\dot{M}_{sum}$ (Vézina et al., 2007; Swanson et al., 2013), we predicted that compared with 'control' birds, 'clipped' chickadees would develop larger pectoral muscles, leading to an increase in $\dot{M}_{sum}$. Assuming that upregulating $\dot{M}_{sum}$ would also require an increase in oxygen delivery (Carey and Morton, 1976; Swanson, 1990b), we expected a positive relationship between haematocrit and $\dot{M}_{sum}$ and higher haematocrit in clipped birds relative to controls.
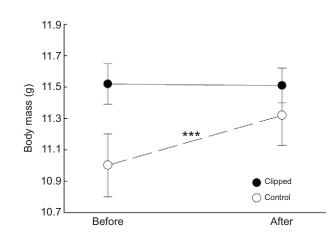
RESULTS
Treatment effect
On the day of first capture, none of the dependent variables we investigated differed among future experimental treatments (group effect: $P=0.1–0.8$). Similarly, individuals that were recaptured later did not differ in any of these variables at first measure from individuals that were never seen again ($P=0.5–0.9$).

Average body mass was not affected by size ($P=0.2$) and did not differ between groups ($P=0.1$), but did vary with time of capture ($F_{1,78}=5.6$, $P<0.05$) and period ($F_{1,69}=5.8$, $P<0.05$). The interaction term period × group was also close to significance ($F_{1,68}=3.4$, $P=0.07$), indicating a trend where control individuals gained body mass between periods while clipped birds did not (Fig. 2). Independent analyses per group confirmed this trend, showing that control birds were on average 2.9% heavier after the treatment than before ($F_{1,45}=15.0$, $P<0.001$), while clipped birds maintained a constant body mass throughout the winter ($P=0.9$; Fig. 2).

Fat scores did not vary between periods ($P=0.5$) or groups ($P=0.09$) and were not affected by the period × group interaction ($P=0.7$).

Analysis of pectoral muscle score showed that both groups ($\chi^2=6.1$, d.f.=1, $P<0.05$) and periods ($\chi^2=5.7$, d.f.=1, $P<0.05$) were significant but that the group effect also tended to depend on the period (period × group: $P=0.07$). Clipped birds had larger muscles on average (median muscle score=3) than controls (median=2). However, when considering the interaction trend, at first capture both control and clipped individuals expressed a median muscle score of 2. After the treatment, clipped birds had larger pectoral muscles (median=3) compared with control birds measured either before or after the treatment (median=2) (Fig. 3). Therefore, the

significant group effect seemed driven mainly by an increase in pectoral muscle size in the clipped group between periods.

Whole $\dot{M}_{sum}$ was influenced by period ($F_{1,51}=38.5$, $P<0.0001$) and group ($F_{1,24}=5.4$, $P<0.05$), with $\dot{M}_{sum}$ being 16.7% higher after the treatment than before and with clipped birds having a $\dot{M}_{sum}$ 7.8% higher than control birds (no significant interaction, $P=1.0$). However, when body mass was included in the model ($F_{1,33}=12.8$, $P<0.01$) there was no difference between groups in $\dot{M}_{sum}$ ($P=0.2$). Mass-independent $\dot{M}_{sum}$ was 14.7% higher after treatment than before (period: $F_{1,58}=29.9$, $P<0.0001$; Fig. 4A).

Haematocrit was not affected by group ($P=0.5$) or the interaction period × group ($P=0.4$), but was dependent on period ($F_{1,76}=38.2$, $P<0.0001$). Birds expressed an average haematocrit 8.5% higher after treatment than before (Fig. 4B). Haematocrit was independent of body mass ($P=0.7$).

Effect of pectoral muscle size and haematocrit on metabolism
When including haematocrit and muscle scores in linear mixed effect (LME) models, variations in mass-independent $\dot{M}_{sum}$ were still affected by period ($F_{1,69}=4.3$, $P<0.05$, 6.9% higher after manipulation) and remained independent from the group effect ($P=0.7$). However, mass-independent $\dot{M}_{sum}$ was positively related to the haematocrit level ($F_{2,69}=10.1$, $P<0.0001$; Fig. 5) and was affected by muscle score ($F_{2,69}=4.1$, $P<0.05$). Therefore, for any experimental group, birds with higher haematocrit and larger pectoral muscles had a higher $\dot{M}_{sum}$ on average than individuals with lower values of both variables. Specifically, compared with individuals with a muscle score of 1, $\dot{M}_{sum}$ was 14.4% higher in birds with a muscle score of 2 (Tukey, $P<0.05$) and 16.5% higher in birds with a muscle score of 3 (Tukey, $P<0.05$) (Fig. 6). However, studying intra-individual changes in muscle score for each group revealed that over the study period, 50% of the clipped individuals (seven individuals out of 14) increased their pectoral muscle to a median score higher than 2 compared with only 29% (five individuals out of 17) for the control birds (Fig. 7). Thus, although the group effect was not significant for $\dot{M}_{sum}$ in this particular analysis, the clipped group contained more
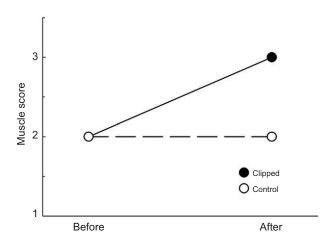
Fig. 3. Variation in muscle score over the experimental period. Data are median muscle scores for control and clipped black-capped chickadees measured before and after the treatment (as these are median values, there are no error bars).

individuals with the highest muscle score during the after period.

DISCUSSION

The seasonal increase in thermogenic capacity commonly reported for wintering birds is often interpreted as the result of an enlargement of shivering muscles (Cooper, 2002; Saarela and Hohtola, 2003; Vézina et al., 2007; Vézina et al., 2011). This can be associated with an elevated haematocrit (Swanson, 1990b) interpreted as an upregulation of oxygen carrying capacity. However, evidence linking muscle size to $\dot{M}_{sum}$ remains correlative (Cooper, 2002; Vézina et al., 2006; Swanson et al., 2013) and the link between haematocrit and $\dot{M}_{sum}$ has not been demonstrated. In this study, we experimentally enlarged pectoral muscle size by clipping flight feathers and observed that: (1) birds with larger pectoral muscles, including a high proportion of clipped individuals, also had high $\dot{M}_{sum}$ values and (2) for a given body mass and muscle size, haematocrit was indeed positively related to $\dot{M}_{sum}$. Results of our phenotypic manipulation therefore support previous correlative evidence for the role of modulating pectoral muscle size and oxygen carrying capacity in $\dot{M}_{sum}$ adjustments.

On average, birds from all treatments increased their body mass, the size of their pectoral muscles, their $\dot{M}_{sum}$ and their haematocrit throughout the experiment. These seasonal changes are part of the natural acclimatization response found in wintering black-capped chickadees. Indeed, both Sharbaugh (Sharbaugh, 2001) and Liknes and Swanson (Liknes and Swanson, 2011) showed increases in body mass in wintering chickadees relative to their summer counterparts. This species is also known to improve its metabolic performance in winter relative to summer (Cooper and Swanson, 1994; Lewden et al., 2012; Petit et al., 2013). For instance, using birds from the same population, Petit et al. (Petit et al., 2013) reported that chickadees elevated their winter $\dot{M}_{sum}$ by 25% between the beginning (October) and the peak of winter (February). The observed development of pectoral muscle size is also part of the natural change in phenotype associated with winter acclimatization, and has been reported in several species (Swanson, 1991b; O'Connor, 1995; Cooper, 2002; Liknes and Swanson, 2011), including black-capped chickadees (Liknes and Swanson, 2011).

In contrast with control birds that showed an increase in body mass during the experiment, clipped individuals maintained a
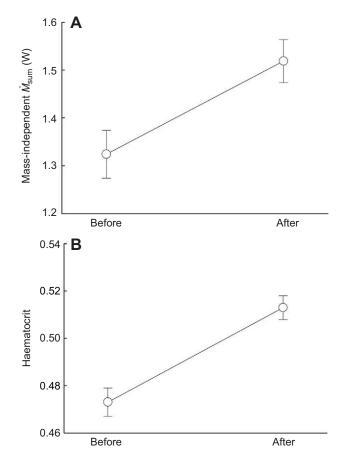

Fig. 4. Changes in thermogenic capacity and haematocrit in black-capped chickadees over the experimental period. Data are least square means of (A) summit metabolic rate ($\dot{M}_{sum}$) and (B) haematocrit before and after the treatment. Least square means were extracted from an LME model controlling for group, period and the interaction term group × period, with bird ID as random parameter. Body mass was also included in the model as a covariate for $\dot{M}_{sum}$.

constant body mass. They also had larger pectoral muscles than control birds, especially during the after period. Therefore, our feather manipulation not only succeeded in forcing clipped individuals to develop larger pectoral muscles, but it also appears that it prevented these birds from increasing their body mass, unlike the controls. One could argue that clipped individuals were already at an optimal body mass at first capture, explaining why we could not detect an increase in mass in this group. However, there was no significant difference in body mass between groups at first capture, which mainly took place in the early autumn (September–October). Given that chickadees typically show a seasonal fattening cycle (Lehikoinen, 1987), peaking at the coldest time of winter [i.e. February at our study site (Petit et al., 2013)], this is therefore very unlikely. Two potential causes could explain the lack of increase in body mass in clipped individuals. First, these birds could have faced elevated daily energy expenditure due to higher wing loading and could therefore have been unable to maintain the positive daily energy budget required for the seasonal fat accumulation (Lehikoinen, 1987). As the number of recaptures was lower in the clipped group, individuals unable to maintain a balanced energy budget could have died as a result of the experiment or moved away to more profitable or less risky habitats. Second, clipped individuals could have maintained
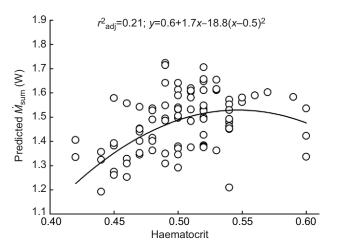
$r^2_{adj}=0.21; y=0.6+1.7x-18.8(x-0.5)^2$

Fig. 5. Relationship between $\dot{M}_{sum}$ and haematocrit in black-capped chickadees. Data are predicted $\dot{M}_{sum}$ values plotted against haematocrit. Predicted values were computed from a LME model controlling for group, period, the interaction term group × period, muscle score and body mass as covariates, with bird ID as a random parameter.

Fig. 6. Relationship between $\dot{M}_{sum}$ and pectoral muscle score in black-capped chickadees. $\dot{M}_{sum}$ data are least square means extracted from an LME model controlling for group, period, the interaction term group × period, body mass and haematocrit as a covariates, with bird ID as random parameter. Different letters indicate significant differences ($P<0.05$).

constant body mass throughout winter as a consequence of higher wing loading, in order to minimise flight costs and maintain manoeuvrability (Pennycuick, 1975; Lindström et al., 2000; Lind and Jakobsson, 2001; Dietz et al., 2007).

The positive relationship between pectoral muscle size and maximal thermogenic capacity confirms previous findings (O'Connor, 1995; Marjoniemi and Hohtola, 1999; Cooper, 2002; Vézina et al., 2007; Swanson et al., 2013). However, this is the first experimental demonstration that muscle size manipulation leads to significant changes in thermogenic capacity and, likely, cold tolerance (Swanson, 2001). Our findings therefore suggest that flight feather clipping could be used as a tool, not only to manipulate muscle phenotypes in small birds (Lind and Jakobsson, 2001), but also to manipulate heat production capacity. However, it is interesting to note that the relationship we observed between $\dot{M}_{sum}$ and pectoral muscle score plateaued at intermediate levels of muscle size (Fig. 6). $\dot{M}_{sum}$ increased by 14–16% when comparing individuals with muscle score 1 to birds with muscle scores 2 and 3, but did not differ significantly between individuals expressing these latter two muscle sizes. This contrasts with previous observations of linear correlations between muscle size and $\dot{M}_{sum}$ (Vézina et al., 2007; Swanson et al., 2013), and suggests that chickadees developing their flight muscles to a size scored as level 3 would pay an unnecessary maintenance cost because it does not provide additional thermogenic capacity. However, one has to keep in mind that, in this experimental setting, the muscle phenotype responded to feather clipping and therefore to a loss of wing and tail lift rather than temperature. Thus, the effect of our manipulation on $\dot{M}_{sum}$ likely resulted from a thermogenic side effect of increased muscle size for locomotor activity (Swanson, 1995; Swanson and Dean, 1999; Vézina et al., 2007). It could be that the relationship between muscle output for locomotion and for shivering is not linear and that, in black-capped chickadees, muscle sizes corresponding to scores 2 and 3 provided a similar heat production capacity. Alternatively, despite the fact that muscle score does reflect pectoral muscle mass (M.P. and F.V., unpublished data; see Materials and methods), measuring muscle size using scores may not be precise enough to detect effects on heat production capacity in individuals expressing relatively large pectoral muscles. Linear measurements such as
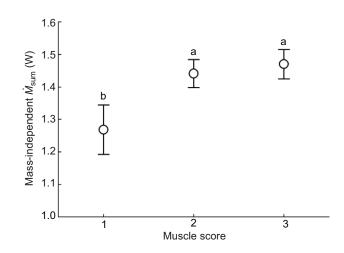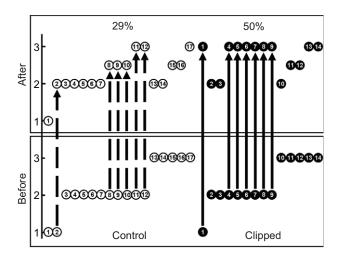
ultrasound scanning (Dietz et al., 1999; Swanson and Merkord, 2013) could improve precision in further studies.

Cold acclimatization is often associated with an increase in haematocrit level (deGraw et al., 1979; Swanson, 1990b; O'Connor, 1996), which is considered a response to elevated oxygen demands for thermogenesis (Carey and Morton, 1976; Swanson, 1990b). Our results are consistent with this interpretation. Haematocrit levels were not affected by group (clipped or control), contrary to what we predicted, but levels were higher after treatment, between January and March, which includes the coldest months of winter. For a given body mass and pectoral muscle size, birds with higher haematocrit levels also expressed a higher $\dot{M}_{sum}$, and this relationship appeared to plateau when haematocrit reached levels around 0.53. Therefore, the pattern of the relationship between haematocrit and $\dot{M}_{sum}$ seemed to parallel that observed between pectoral muscle size and $\dot{M}_{sum}$. The parabolic shape in the relationship between $\dot{M}_{sum}$ and haematocrit is consistent with the idea that beyond a certain level, the benefit of increasing oxygen carrying capacity is counterbalanced by the disadvantage of elevated blood viscosity (Schuler et al., 2010; Williams et al., 2012). Therefore, it could be that chickadees have an optimal haematocrit level that maximizes their heat production capacity and cold endurance, and given that heart mass fluctuates seasonally in small wintering passerines (e.g. Zheng et al., 2008), including chickadees (Liknes and Swanson, 2011; M.P., A. Lewden and F.V., unpublished data), this optimal level is likely to change throughout the year. As far as we know, this is the first demonstration of the relationship between maximal thermogenic capacity and haematocrit in birds. However, this link remains correlative, and experimental manipulations of haematocrit levels (Williams et al., 2012) should be conducted to confirm its role in supporting thermogenesis.

MATERIALS AND METHODS
Capture and handling
Black-capped chickadees are small (9–14 g) non-migratory passerines that form territorial social groups during winter (Smith, 1993), which allows for tracking individuals over time relatively easily. This study was carried out in the Forêt d'Enseignement et de Recherche Macpès, Québec, Canada (48°30′N, 68°52W), where we caught 60 free-living individuals between

Fig. 7. Individual changes in muscle scores between periods. The percentage of black-capped chickadees increasing their muscle size to a score higher than 2 is presented above each group. Muscle scores presented for the after period are median values of all scores measured for a given bird over all its recaptures. Therefore, values for this period may include decimals. Arrows highlight birds that increased their muscle score between periods.

September 2010 and March 2011 (average minimal temperature recorded hourly over the study period: −2.5±0.1°C; see Table 1 for details). Eighteen feeding stations were set up, each including three metal feeders filled with black sunflower seeds and two wooden poles that were topped with plastic trays (Lewden et al., 2012; Mandin and Vézina, 2012). On capture days, we removed feeders and put a potter-trap (15×15×15 cm) baited with black sunflower seeds on each tray. All birds were caught between 08:00 and 13:00 h and, following capture, birds were banded with a USGS numbered metal band and a unique combination of three coloured plastic bands that allowed individual identification from a distance.

For each captured bird, we collected a blood sample within less than 10 min by puncturing the brachial vein with a 26-gauge needle. Samples (total blood volume <120 µl) were kept cold (<4°C) in heparanized microcapillary tubes until centrifugation (10 min at 16,000 *g*) and haematocrit measurements. After blood sampling, birds were weighed and were measured following standardized protocols (length of beak, head plus beak, tarsus, tail and wing measured) (Lewden et al., 2012; Mandin and Vézina, 2012; Petit et al., 2013). The furcular fat stores were evaluated using a visual score from 0 (no visible fat) to 5 (fat overlapping pectoral muscles) according to Gosler (Gosler, 1996). Pectoral muscle size was determined on a scale of 0 (keel prominent and muscles depressed) to 3 (keel difficult to see and muscles rounded) (Busse, 2000). Although muscle score is a relatively crude index, it nevertheless reflects differences in pectoral muscle mass. Indeed, we dissected pectoral muscles of 79 chickadees for another study and observed a positive relationship between muscle score and size-corrected lean dry mass of pectoral muscles ($F_{2,75}$=29.2, P<0.0001, r^2_{adj}=0.45). Birds with muscles scored as 3 had pectoral muscle on average 21% heavier than birds scored as 2, and these latter individuals had pectoral muscles 18% heavier than those scored as 1 (M.P. and F.V., unpublished data).

Table 1. Mean (±s.e.m.) minimal ambient temperature (T_a) recorded hourly over the study period

Month	Mean minimal T_a (°C)
September	11.44±0.19
October	05.15±0.14
November	−01.02±0.15
December	−05.07±0.19
January	−11.20±0.25
February	−11.52±0.23
March	−05.76±0.23

The black-capped chickadee is a monomorphic species. We were therefore unable to determine the sex of individuals for this study. For each day of capture, a maximum of four birds were brought to the station for $\dot{M}_{sum}$ measurements. All bird manipulations were approved by the animal care committee of the Université du Québec à Rimouski and have been conducted under scientific and banding permits from Environment Canada – Canadian Wildlife Service.

Respirometry
At the station, birds were maintained in individual cages (39 cm×43 cm×31 cm) with food (sunflower seed) and water provided *ad libitum* prior to $\dot{M}_{sum}$ measurements. Within 24 h of capture, we measured their $\dot{M}_{sum}$ between 13:00 and 18:00 h and released the individuals at their original catching site after having performed the experimental manipulation (see below).

We measured the $\dot{M}_{sum}$ of two birds at the same time using two FoxBox oxygen analyzers (Sable Systems, Las Vegas, NV, USA) and following the protocol described by Lewden et al. (Lewden et al., 2012) and Petit et al. (Petit et al., 2013). Before the $\dot{M}_{sum}$ measurement, we weighed (±0.1 g) each individual and measured their body temperature with a copper–constantan thermocouple inserted into the cloacae ~10 mm deep (NIST-traceable thermocouple reader Omega model HH-25KC, Montréal, QC, Canada). Birds were then placed in a stainless steel metabolic chamber (effective volume=1120 ml) and received a mixture of 21% oxygen and 79% helium (helox) using an average flow rate of 1109 ml min^{-1} controlled by mass flow valves (Sierra Instruments, Side-Trak Model 840, Monterey, CA, USA). We used a sliding cold exposure protocol (Swanson et al., 1996) during the $\dot{M}_{sum}$ trials, where ambient temperature was ramped down by 3°C every 20 min, starting at 0°C. Trials ended when birds became hypothermic, which was easily detectable from the O_2 readings. Body temperature and body mass were measured again at the end of each trial. Only data from birds showing a body temperature after trials lower or equal to 38°C (Cooper and Gessaman, 2005) were used and average body mass was used in the statistical analyses. $\dot{M}_{sum}$ values were calculated using the highest averaged 10 min of oxygen consumption calculated with the instantaneous measurement technique (Bartholomew et al., 1981). Because birds use lipids as metabolic fuel during shivering (Swanson, 2010), we estimated energy consumption in Watts using a constant equivalent of 19.8 kJ l^{-1}O$_2$ (Gessaman and Nagy, 1988).

Mass flow valves were calibrated for helox with a bubble-O-meter (Dublin, OH, USA) prior to the experiment, and oxygen analyzers were adjusted before each $\dot{M}_{sum}$ trial with CO$_2$-free dry air. Calculations of metabolic rates were made using EXPEDATA software, v1.2.6 (Sable Systems, Las Vegas, NV, USA), according to eqn 10.1 from Lighton (Lighton, 2008).

Experimental treatments
From September to December 2010, we used a feather clipping protocol to manipulate pectoral muscle size. Following respirometry measurements, we randomly assigned birds to one of two experimental treatments: 'clipped' or 'control'. Clipped birds had half of their flight feathers cut along the main shaft (i.e. removing barbs and barbules) on both wings (primaries P1, P3, P5, P7 and P9) and both sides of the tail (rectrices R2–R4). This approach prevents premature regrowth of cut feathers (P. Perret, personal communication) and ensures long lasting effects of the treatment until the next molt (Sanz et al., 2000; Harding et al., 2009a). Control individuals were handled similarly to clipped birds, but their feathers were not clipped. Following $\dot{M}_{sum}$ measurement, half of the birds were assigned to the clipped group while the other half was assigned to the control group. Over the 60 birds (clipped: N=29; control: N=31) initially caught and measured, we obtained a final sample size of 14 clipped and 17 control individuals (recapture rate=55% for control birds and 48% for clipped birds) (Fig. 1).

Statistical analysis
Testing for the effect of duration between captures and recaptures
This protocol was carried out in natural conditions and this resulted in variable numbers of recaptures per individual (Fig. 1). However, there was no difference in the average number of recaptures between treatments

(clipped: 1.9±0.4, control: 2.7±0.4; $P=0.1$), and the average duration between first capture (i.e. the day of manipulation) and all recaptures for a given bird also did not differ between treatments (79.6±10.4 days for clipped birds and 84.1±7.5 days for control birds; $P=0.3$). To determine whether the duration between captures could create a significant effect on our dependent variables, we used data from individuals for which we had at least one recapture and tested for an effect of the duration between the day of manipulation and the day of each capture. This was done using LME models for linear variables (body mass, haematocrit and $\dot{M}_{sum}$) and ordinal random effect regressions (Christensen, 2013) for ordinal parameters (fat and muscle scores). Models tested for the effect of duration while controlling for date of capture, group (clipped or control) and the interaction group × duration, with bird ID as a random parameter. However, $\dot{M}_{sum}$ is affected by body mass and body mass is affected by structural body size and daytime because of daily fattening (Mandin and Vézina, 2012). We therefore performed a principal component analysis on morphological data (length of head plus beak, wing and tarsus) so that we could use the first principal component as a measure of structural body size (hereafter called 'size') (Rising and Somers, 1989), and we also calculated relative time (time since sunrise/day length, hereafter 'time of capture') for each capture. We could then analyse the effect of the duration between captures on size-independent body mass and mass-independent metabolism by adding size and time of capture or mass as covariates in LME models. In all of these analyses, we used a likelihood ratio test (LRT) to compare the complete models with models without the effect of duration between captures. Duration had no significant effect on any of our dependent variables (size-independent body mass: $P=0.5$; haematocrit: $P=0.6$; mass-independent $\dot{M}_{sum}$: $P=0.2$; fat score: $P=0.2$; muscle score $P=0.9$). We therefore did not include duration between captures in further analyses and rather encoded our data according to two periods. The period called 'before' designates data collected at the first capture (before applying the treatment, between September and December with 62% of the first captures made between September and October) while the period called 'after' designates all measurements collected each time a bird was recaptured (individuals were recaptured from one to seven times with an average of 2.3±0.3 times between October and March; 59% of all the recaptures happened between January and March). Because date of capture was redundant with period, we also removed the variable 'date of capture' from further analyses.

Testing for treatment effects on dependent variables

We began our analyses by testing whether control and clipped individuals were different at first capture (i.e. before manipulation, $N=60$). We used general linear models or ordinal regressions to test for a group effect on body mass, haematocrit, $\dot{M}_{sum}$, fat score and muscle score while considering the potential effect of date and time of first capture as well as body size or body mass when appropriate (see above). We then investigated whether individuals that were later recaptured differed in these variables relative to birds that were not recaptured. This was done by including the variable 'recapture' (yes or no) in the models.

To test for the treatment effect, only data from birds that were recaptured at least once were used. Fat and muscle scores were analysed by ordinal random effect regressions to track changes in fat stores and muscle size over the winter. Ordinal regressions considered the effects of period (before or after treatment), group and the interaction term period × group, with bird ID as a random parameter to account for repeated measures. The effect of each variable was determined using the LRT method (Christensen, 2013). Fat and muscle scores were measured by four observers (66% of the observations made by M.P.) but when considered in models, the effect of observers on fat score and muscle score was not significant ($P=0.1$ and 0.3, respectively). We therefore did not consider this effect in our analyses.

To analyse winter variations of body mass, $\dot{M}_{sum}$ and haematocrit according to period, group and the interaction term period × group, we ran LME models including bird ID as a random variable. We controlled for the effect of size and time of capture on body mass and mass on $\dot{M}_{sum}$ by including these variables as covariates in respective models. Muscle score and haematocrit were also added to the $\dot{M}_{sum}$ model to study the effect of pectoral muscle size on mass-independent $\dot{M}_{sum}$ and the potential relationship between haematocrit and thermogenic capacity. Because we

expected a parabolic relationship between $\dot{M}_{sum}$ and haematocrit, we considered the quadratic relationship between these variables by including a second-order polynomial function for the haematocrit effect in the LME.

Ordinal regressions were performed with R (R Core Team, 2013) using the ordinal package (Christensen, 2013). *Post hoc* analyses were performed using Tukey tests on least square means computed from LMEs. We removed non-significant variables from models and final results are presented here as least square means ± s.e.m. for linear parameters and as medians for ordinal variables. Residuals were tested for normality using the one-sample Kolmogorov–Smirnov test.

Author contributions
M.P. and F.V. designed the experiment; F.V. provided materials; M.P. performed the experiments and analyzed the data; M.P. and F.V. wrote the manuscript.

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