Am J Physiol Regul Integr Comp Physiol 318: R274–R283, 2020.
First published December 11, 2019; doi:10.1152/ajpregu.00203.2019.

RESEARCH ARTICLE | *Obesity, Diabetes and Energy Homeostasis*

Consequences of being phenotypically mismatched with the environment: rapid muscle ultrastructural changes in cold-shocked black-capped chickadees (*Poecile atricapillus*)

François Vézina,[1] Emily Cornelius Ruhs,[1] Erin S. O'Connor,[2] Audrey Le Pogam,[1] Lyette Régimbald,[1] Oliver P. Love,[3] and Ana Gabriela Jimenez[2]

[1]*Département de Biologie, Chimie et Géographie, Groupe de Recherche sur les Environnements Nordiques BORÉAS, Centre d'études Nordiques, Centre de la Science de la Biodiversité du Québec Université du Québec à Rimouski, Québec, Canada;* [2]*Department of Biology, Colgate University, Hamilton, New York; and* [3]*Department of Integrative Biology, University of Windsor, Ontario, Canada*

Submitted 8 July 2019; accepted in final form 4 December 2019

Vézina F, Cornelius Ruhs E, O'Connor ES, Le Pogam A, Régimbald L, Love OP, Jimenez AG. Consequences of being phenotypically mismatched with the environment: rapid muscle ultrastructural changes in cold-shocked black-capped chickadees (*Poecile atricapillus*). *Am J Physiol Regul Integr Comp Physiol* 318: R274–R283, 2020. First published December 11, 2019; doi:10.1152/ajpregu.00203.2019.—Phenotypic flexibility has received considerable attention in the last decade; however, whereas many studies have reported amplitude of variation in phenotypic traits, much less attention has focused on the rate at which traits can adjust in response to sudden changes in the environment. We investigated whole animal and muscle phenotypic changes occurring in black-capped chickadees (*Poecile atricapillus*) acclimated to cold (−5°C) and warm (20°C) temperatures in the first 3 h following a 15°C temperature drop (over 3 h). Before the temperature change, cold-acclimated birds were consuming 95% more food, were carrying twice as much body fat, and had 23% larger pectoralis muscle fiber diameters than individuals kept at 20°C. In the 3 h following the temperature drop, these same birds altered their pectoralis muscle ultrastructure by increasing the number of capillaries per fiber area and the number of nuclei per millimeter of fiber by 22%, consequently leading to a 22% decrease in myonuclear domain (amount of cytoplasm serviced per nucleus), whereas no such changes were observed in the warm-acclimated birds. To our knowledge, this is the first demonstration of such a rapid adjustment in muscle fiber ultrastructure in vertebrates. These results support the hypothesis that chickadees maintaining a cold phenotype are better prepared than warm-phenotype individuals to respond to a sudden decline in temperature, such as what may be experienced in their natural wintering environment.

capillaries per fiber area; muscle fiber diameter; muscle ultrastructure; myonuclear domain; phenotypic mismatch

INTRODUCTION

Phenotypic flexibility is a phenomenon by which adult organisms reversibly adjust their physiological or behavioral traits in response to environmental constraints (34, 35). This concept has received considerable attention in the last decade (see Ref. 34 for an extensive review) and several studies have shown remarkable examples of variation in trait value across life-history stages (e.g., 33, 35, 59).

Although reporting amplitude of variation in phenotypic traits is useful and increasingly common (e.g., Refs. 22, 23, 35, and 59), much less attention has been devoted to the rate at which traits can adjust in response to sudden changes in the environment (e.g., Refs. 1, 10, and 49). Yet, the rate of change should be as critical as trait amplitude, especially if a given phenotype is mismatched to the environment (24, 56). For example, McWilliams and Karasov (24) showed that white-throated sparrows (*Zonotrichia albicollis*) previously acclimated to 21°C and exposed to −5°C or −20°C for 2 days were only able to increase their food consumption by 45%–57% above that preceding the temperature drop. This limited increase in food intake was considered to reflect the "immediate spare capacity" of the digestive system, and, as it did not match the energy demand at the new temperatures, birds showed negative energy balance and lost body mass. In contrast, individuals given 12 days to acclimate to these same cold temperatures were able to meet the demand, as they maintained their body mass while eating 24%–26% more food on average than the cold-shocked birds. In another example, Dubois et al. (10) showed that summit metabolic rate (M_{sum}), a measure of maximal shivering heat production seen as an index of avian cold endurance (20, 44), responded very slowly to a sudden drop in ambient temperature. In fact, although cold acclimation is known to lead to higher M_{sum} in each of these model species (2, 30), none of black-capped chickadees (*Poecile atricapillus*), snow buntings (*Plectrophenax nivalis*), or white-throated sparrows showed significant increase in cold endurance in the first 8 days following a 15°C drop from an acclimated state at 10°C, despite a clear increase in thermoregulatory demand showed by daily food intake.

Given the potential delay in response to rapid environmental changes, animals certainly benefit from maintaining some level of reserve capacity in life-sustaining phenotypic traits such as digestive or thermogenic capacity (24). However, as adjusting reserve capacity in slow-responding traits such as M_{sum} may require preparation ahead of time (30), the amount of reserve capacity maintained at any time may also depend on specific

Address for reprint requests and other correspondence: F. Vézina, Département de biologie, chimie et géographie, Université du Québec à Rimouski, 300 Allée des Ursulines Rimouski, Québec G5L 3A1 Canada (e-mail: francois_vezina@uqar.ca).

phenotypes. For instance, Petit and Vézina (32) showed the reaction norm for M_{sum} in free-living black-capped chickadees over a complete seasonal cycle, in which birds began to increase their thermogenic capacity as temperatures declined in the fall to reach a plateau in M_{sum} when mean minimal temperature was around −10°C (i.e., in November to December; 32). This suggests that the phenotype expressed at this time likely had enough reserve capacity to allow birds to endure cold spells typical of later in the season; that is, short-term declines in temperature by 10°C–20°C (our source population is exposed to extreme temperatures in January to March that can reach ≤−25°C) (32). The counterpart of this is that birds experiencing similar declines in temperature in summer or during the transition seasons (fall and spring) would presumably be less prepared and would undergo a higher proximate cost if they become phenotypically mismatched.

Shivering heat production is critical for thermoregulation and winter survival in small birds (18, 29), and shivering is ultimately underscored by muscle physiology (47, 49–51). Indeed, previous studies have shown positive correlations between muscle mass and M_{sum} or between directional changes in these two variables (10, 25, 50, 52, 58). However, accumulating evidence also suggests that although maintaining a large mass of skeletal muscles might be advantageous in terms of total heat production, an actual increase in muscle size may not always be vital to improve cold endurance or could be context specific (2, 12, 25, 42, 52). Nonetheless, the exact mechanisms involved in adjusting thermogenic capacity are not clear, although available data increasingly point toward cellular mechanisms (2, 7, 25, 42).

In this context, an aspect of skeletal muscle physiology that has received little attention is muscle ultrastructure. Muscle fiber size can influence whole animal metabolism (13, 16). For example, small-diameter fibers have lower diffusion distances for metabolites such as O_2 and ATP, which is beneficial for high-metabolic activities such as shivering (16), and small muscle fibers have been reported in cold-acclimated pigeons (*Columba livia*) (21). Conversely, large muscle fiber diameters can also be advantageous since large fibers produce higher contraction force, which presumably increases shivering heat production. They also have lower basal metabolic costs as a result of decreases in surface area per unit volume and ATP-consuming, membrane-bound processes (13, 16). Given that total muscle mass can significantly impact whole animal basal metabolic rate in birds (6, 25, 57), maintaining large fibers could therefore be beneficial in reducing overall maintenance energy demand (47). Black-capped chickadees coming out of winter (April to May) have been found to maintain larger fibers in pectoralis muscles than individuals caught in summer (July to August) (15). Direct corollaries of muscle fiber function are myonuclear domain and the number of capillaries per fiber area. Myonuclear domain is defined as the amount of cytoplasm serviced per nucleus (36) and is often positively correlated with fiber diameter (14). The number of capillaries per unit surface area is also a key trait in active muscles since capillaries provide oxygen and fuel to fibers. Previous studies showed high capillary density in cold-acclimated pigeons (21) and spring chickadees (15), and recent work on captive cold-acclimated dark-eyed juncos highlighted upregulation of muscle genes involved in angiogenesis (42). As these muscle traits are typically studied by comparing stable phenotypes, it is unclear, however, how quickly these features (i.e., cellular muscle phenotype) can change in response to sudden environmental change.

In this study, we examined captive black-capped chickadees maintained under cold winter-like (−5°C) and warm fall-like (20°C) conditions, and we investigated how birds maintaining stable phenotypes under these conditions responded to a sudden 15°C drop in temperature in terms of daily food intake, fat mass, and lean mass as well as muscle ultrastructure. Experimental temperatures were chosen after the reaction norm measured for M_{sum} by Petit et al. (32, Fig. 1). We assumed that the reaction norm for M_{sum} would be a good approximation of the phenotypic changes expected in all measured traits given the importance of M_{sum} for winter survival (18, 29), its clear response to temperature (46, 49, 62), and the fact that both M_{sum} variation, which results from muscle physiological changes (25, 42, 51, 57, 62–64), and rate of food consumption, which forces digestive adjustments (2, 24), are slow or limited in their response to rapid temperature changes (10, 24). The first thermal environment, at −5°C, was equivalent to midwinter in our temperate population, for which the drop in temperature should have a minimal impact, as birds would be expected to buffer the demand with spare capacity (Fig. 1). We therefore expected these individuals to consume large amounts of food and to maintain large fat reserves and a high lean mass (31). We also expected to find relatively large fiber diameters with large myonuclear domains within their pectoralis muscles and high counts of capillaries per fiber area (15). We further predicted that these birds would increase their food intake and show minimal loss in fat and lean mass or muscle ultrastructure in response to the change in temperature. In the second environment, birds were kept at 20°C, a temperature equivalent to early fall in our population. We expected these birds to maintain smaller fat reserves and lean mass, eat less, and maintain smaller muscle fiber size, with small myo-

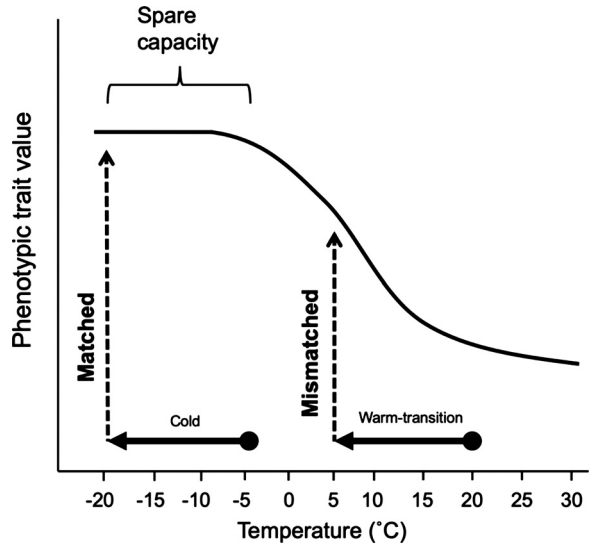

Fig. 1. Schematic representation of the reaction norm for phenotypic traits thought to covary with summit metabolic rate in response to ambient temperature (°C). Vertical dashed lines represent the matched (cold) and mismatched (warm-transition) phenotypes. The solid arrows represent the temperature drop for each group (15°C each). Spare capacity is represented by the absence of change in a phenotypic trait for a given change in ambient temperature.

nuclear domains, surrounded by fewer capillaries than cold-acclimated individuals. In these birds, the 15°C decline in temperature should bring individuals to the equivalent of early winter, which normally requires a transition to a new phenotype during seasonal acclimatization (Fig. 1; 32). Therefore, as the sudden temperature drop would create a phenotypic mismatch in this group, these warm-transition birds would presumably have less spare capacity for energy intake, and thus be expected to show a decline in fat reserves, a loss of lean mass, and reduced muscle function in the hours following the change in temperature.

MATERIALS AND METHODS

Capture, Maintenance, and Temperature Protocol

Black-capped chickadees were captured near Rimouski, Québec, Canada, in the forêt d'enseignment et de recherche Macpès using mist nets in winter and fall. Birds were then transported back to the Avian Facility of the Université du Québec à Rimouski, where they were fitted with a unique combination of color bands. The birds were housed individually (41.3 × 41.9 × 32.4-cm cages) and were randomly assigned to an experimental group. Procedures were approved by the Université du Québec à Rimouski Animal Care Committee (CPA-69–17–191) and have been conducted under scientific and banding permits from Environment Canada - Canadian Wildlife Service (10889).

We used a factorial design with four treatment combinations. Birds were divided into cold (−5°C, $n = 18$) and warm-transition (20°C, $n = 20$) phenotypes and were held in these conditions for 21 days before the temperature change. These two phenotypes were further divided into a control group, experiencing no temperature change, and a treatment group, which would be exposed to a decrease in temperature by 15°C over 3 h at a rate of 5°C/h. As the starting temperatures for each phenotype occur at different times of the year, birds were also exposed to photoperiods matching these periods for the location of our source population. Cold-acclimated birds were kept under a 10 h, 15 min light:13 h, 45 min dark photoperiod (i.e., February), whereas the warm-transition birds were under a 13 h, 23 min light:10 h, 37 min dark cycle (i.e., September).

Measured Variables

For 3 days (i.e., 3 24-h periods) before the temperature drop, 24-h food intake was measured for all birds and converted into kJ/day (8). Briefly, birds were given a measured amount of deshelled-sunflower seed (~20 g) and mealworms (~0.6 g; mealworms were always consumed rapidly), and orts were measured on the following morning. Using data on water content of seeds and mealworms and metabolizable energy content, we determined the kilojoules per day consumed per bird (8). Two days (starting on *day −2*) before the scheduled temperature drop, we also measured food intake for all birds from 8:00 to 11:00 and from 11:00 to 14:00 (cold) or from 5:51 to 8:51 and from 8:51 to 11:51 (warm transition) (3-h time blocks) to match the time period at which food intake could be measured on the experimental day ("before"). The day before the temperature drop, we also measured total fat and lean mass of all birds by quantitative magnetic resonance (EchoMRI, Houston; values ± 0.01 g) (11). Each bird was measured a minimum of three times (1.5–3.5 min) (11) and average values were used in analyses.

On the experimental day, treatment birds experienced the 15°C temperature decline from 8:00 to 11:00 (cold) or 5:51 to 8:51 (warm-transition), during which we measured their "during" food-intake rate [food given at 8:00 and replaced at 11:00 (cold) or given at 5:51 and replaced at 8:51 (warm-transition)]. At the end of that time block [11:00 (cold) and 8:51 (warm-transition)], birds were again given a measured quantity of food, and orts were weighed at 14:00 (cold) or 11:51 (warm-transition) to obtain the "after" intake rate. Birds then remained at their final room temperatures (20°C and −5°C for warm-transition birds and −5°C and −20°C for cold birds) for 3 h before sampling and measurements. Birds experiencing the temperature drop were therefore exposed to 6 h of cold; a gradual temperature decline in the first 3 h and a new stable temperature in the next 3 h.

After following these procedures, we removed birds from each room by alternating among experimental groups, until each bird had been measured, euthanized, and dissected. We first measured total body weight (± 0.01 g), and lean and fat masses by quantitative magnetic resonance and then proceeded to euthanize birds by CO_2 asphyxiation, after which we extracted pectoralis muscle samples within 5 min of death, which were immediately fixed in 4% paraformaldehyde and shipped to Colgate University for further processing.

Laboratory Analyses

Muscle sectioning and staining. Fixed muscle sections were put in 30% sucrose overnight before sectioning to cryoprotect samples. Each muscle sample was divided in two pieces: one oriented in cross section (for fiber diameter and fiber cross-sectional measurements) and the other oriented longitudinally (to measure longitudinal nuclei length for myonuclear domain determination). Tissues were mounted in optimal cutting-temperature compound (Scigen Scientific) and allowed to equilibrate to −20°C in a Microm HM 505 N cryostat before sectioning. Sections were then cut at 30 μm, picked up on plus slides (Fisher Scientific), and air-dried at room temperature, then stained with a 250 mg/mL solution of wheat germ agglutinin (WGA; Molecular Probes) with Alexa Fluor 488, 4′,6-diamidino-2-phenylindole (DAPI; Molecular Probes) and *Griffonia simplicifolia* lectin (GSL; Molecular probes) 694 for 30 min and then rinsed in avian ringer's for 60 min. WGA can nonspecifically bind to tissue, and thus longer rinse times are necessary. WGA is a lectin that binds to glycoproteins on the basement membrane of the fiber sarcolemma and effectively outlines the fiber periphery to allow measurements of fiber size (26, 60). DAPI binds to nuclei and *Griffonia simplicifolia* lectin II binds to capillaries. Stained slides were examined with a Zeiss 710 laser filter confocal microscope. Polygons were traced along the fiber periphery using Image J (13, 26). Each stain was analyzed using stain's individual channel. Polygons were initially drawn in WGA images in Image J and could be kept in place as the other two stains were quantified. To manually count capillaries and myonuclei, we used split images of each of our stains so that we could identify capillaries and myonuclei independently of the other two stains. We did not use any oblique sections to quantify our parameters, nor did we count fibers that showed any degree of muscle fascicle separation or freeze fracture. Fibers ($n = 45$) were randomly chosen and measured to calculate averages for each individual chickadee.

Myonuclear domain determination. Fiber margins were traced using Image J, and resultant polygons were used to calculate fiber cross-sectional area, whereas nuclear cross-sectional area and diameter (from fiber cross sections) and nuclear lengths (from longitudinal sections) were calculated by outlining DAPI-stained nuclei. Number of nuclei per millimeter of fiber (x) was calculated following the method of Schmalbruch and Hellhammer (40): $x = nL/d + l$, in which n represents the number of myonuclei per cross section of fiber, L is the desired length of the fiber segment (1,000 mm), d is the thickness of the section (30 μm), and l is the mean length of a muscle nucleus measured from longitudinal sections. The volume of cytoplasm per nucleus or myonuclear domain (y) was calculated by $y = CL/x$, in which C is the cross-sectional area of the muscle fiber measured. Nuclear number volume, which is the number of nuclei per volume of cell, is the inverse of the myonuclear domain, y.

Capillaries per fiber area. Fiber cross-sectional area and number of capillaries around the fiber were analyzed using ImageJ. Capillary counts were measured by manually counting the number of capillaries touching the fiber. Capillaries per fiber area (CFAs) were obtained by

dividing the capillary counts by muscle fiber cross-sectional area of each individual fiber (4, 39).

Statistical Analysis

All statistical analyses were run in JMP (Version 13.1.0). We began by creating a first principal component (PC) based on morphological data [head, beak, tarsus, and wing (mm)] to be used as our measure of structural body size in subsequent analyses (38).

For whole organism responses, we then used nested mixed models to examine variation in body mass, fat mass, lean mass, and food intake within phenotypes. We included the categorical predictors phenotype, treatment[phenotype], period[phenotype], treatment × period[phenotype]. Models also included PC size as a covariate and bird ID as a random variable to take into account repeated measures. We defined phenotype here as "cold" or "warm transition," treatment as "temperature drop" or "control," and period as "before" or "after" the temperature drop. For food intake, we did not use the variable period but instead included a parameter called "time" (i.e., the 3 3-h periods of food intake, "before," "during," and "after" the temperature drop, described above). We used Tukey's honestly significant difference post hoc analyses to examine difference among groups for categorical variables. Data are presented as least squares means ± SE.

For muscle ultrastructure, we analyzed the data using general linear models to determine if there were differences between phenotypes or treatments. We thus tested for the effect of phenotype and treatment[phenotype]. Although it is common practice to present unadjusted mean values per experimental groups in histology studies (e.g., Ref. 13), in some circumstances this approach can overshadow important variation, especially if independent variables covary with structural parameters, which can deform or create false differences among experimental groups (see Refs. 27 and 28 for examples). In the present case, total capillaries per fiber area varied negatively, whereas the number of nuclei per millimeter of fiber and myonuclear domain varied positively with fiber diameter (see RESULTS). Therefore, comparing unadjusted means for these variables among groups of birds expressing different fiber diameter, as in this study (see below), would be misleading. We therefore included fiber diameter as a covariate in models examining these variables, which made our models nested analyses of covariance (27, 28), and presented least square means ± SE in figures, thus controlling for the effect of fiber diameter on independent variables. However, for comparisons with other histology studies, we also present unadjusted mean values in all figures. Similar to our whole organism variables, we used Tukey's honestly significant difference post hoc analyses to examine differences among groups for categorical variables.

Although models must include the terms "Treatment[Phenotype]," "Period[Phenotype]," or "Time[Phenotype]" to test for their interactions, these particular statistical effects are not biologically relevant in the context of this study since they pool some of the effects (treatment, period, time) our experiment was designed to investigate. The following results therefore focus on the effects of phenotype and the interaction terms. It should also be noted here that we present nested variables based on their simplest names (e.g., treatment[phenotype] = treatment) for clarity in the following results. Inspection of residuals distribution confirmed that model assumptions were respected.

RESULTS

Final sample sizes for the cold phenotype were nine birds experiencing the temperature drop and nine control birds. Final sample sizes for the warm-transition phenotype were 10 individuals experiencing the temperature drop and 10 control birds.

Whole Organism

Cold-acclimated birds consumed 95% more food on average (6.2 ± 0.3 kJ/h) than birds from the warm-transition group (3.2 ± 0.3 kJ/h; Fig. 2), confirming the higher thermoregulatory demand in this group. The two phenotypes also reacted differently to the temperature drop as shown by a significant interaction term treatment × time (Table 1). Indeed, within the warm-transition phenotype, we observed a decline in food intake while the temperature was declining, but this was apparent in all birds, whether they were experiencing the temperature change or not (Fig. 2). In the following 3 h, the birds had either resumed their pre-experimental food intake (control) or settled to a level statistically intermediate (temperature drop). In cold-acclimated birds, both temperature-drop birds and control birds had increased their food consumption in the last period. However, the increase appeared much faster in birds exposed to declining temperatures, which already had increased their food intake by 49% in the 3 h during which the temperature declined. In the next 3 h, birds were eating 49%

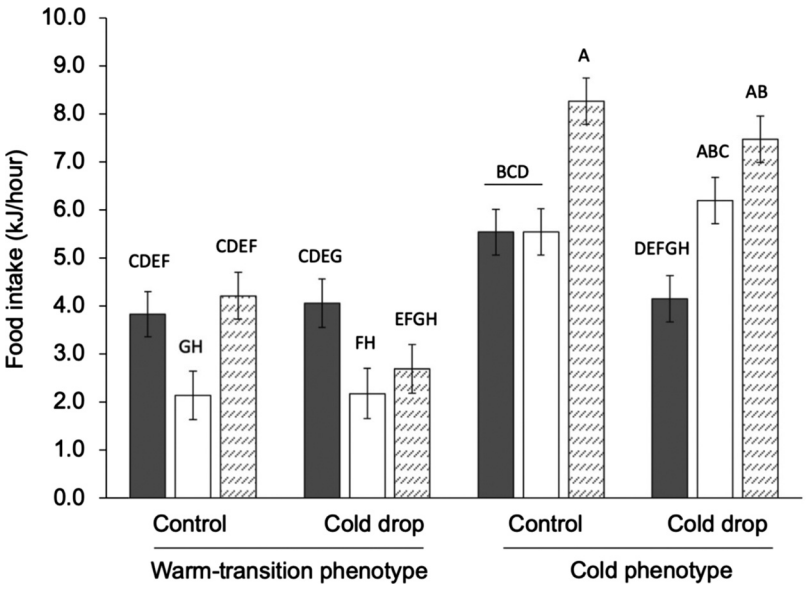

Fig. 2. Food intake (kJ/hour) before (shaded bar), during (open bar), and after an acute temperature drop (hatched bar) for the control and cold-drop groups within each phenotype. Values are least square means ± SE, controlling for the random effect of bird ID. Bar with different letters presents statistically significant differences at $P < 0.05$.

Table 1. *Summary of statistical results for the effect of phenotype and treatment on food intake and body composition of black-capped chickadees*

		Model Statistics		
Measure	Factor	df	*F*	*P*
Food intake	Phenotype	1	52.2	<0.0001
	Treatment[phenotype]	2	0.6	0.54
	Time[phenotype]	4	31.5	<0.0001
	Treatment × time[phenotype]	4	4.9	0.002
Total body mass	Phenotype	1	19.1	<0.0001
	Treatment[phenotype]	2	0.7	0.49
	Period[phenotype]	2	2.6	0.09
	Treatment × period[phenotype]	2	1.0	0.40
	Size	1	34.2	<0.0001
Fat mass	Phenotype	1	22.1	<0.0001
	Treatment[phenotype]	2	0.1	0.93
	Period[phenotype]	2	5.9	0.007
	Treatment × period[phenotype]	2	4.1	0.03
	Size	1	3.0	0.09
Lean mass	Phenotype	1	1.7	0.20
	Treatment[phenotype]	2	1.0	0.39
	Period[phenotype]	2	6.4	0.005
	Treatment × period[phenotype]	2	2.1	0.136
	Size	1	39.8	<0.0001

df, degrees of freedom.

(control) and 80% (temperature drop) more food than on the day before the temperature drop (Fig. 1).

Controlling for the significant influence of body size, total body mass only differed among phenotypes, with cold-acclimated birds being 8.7% heavier on average than warm-transition birds (Table 1, Fig. 3). Cold-acclimated birds also carried twice as much fat (109%) than individuals from the warm-transition group (Table 1). However, within phenotype, fat reserves varied differently in birds experiencing the temperature drop than in those forming the control groups (significant interaction treatment × period) (Table 1). Closer scrutiny of post hoc comparisons revealed that this was due to cold-acclimated birds having increased their fat mass by 34% following the temperature drop, whereas no other experimental groups showed significant changes in fat (Fig. 3). Lean mass was not affected by phenotype when considering the effect of structural body size and the interaction term treatment × period was not significant (Table 1). Therefore, the cold-acclimated phenotype was formed of birds eating more food and carrying more fat than birds from the warm-transition phenotype. These birds were also the only ones significantly increasing food consumption.

Muscle Ultrastructure

Muscle fiber diameter differed with regard to phenotype but not with regard to treatment within phenotype (Table 2). Cold-acclimated birds had 23% larger fibers than birds from the warm-transition phenotype (Table 2, Fig. 4). Fiber diameter was also significantly negatively correlated with capillaries per fiber area ($r^2 = 0.36$, $n = 38$, $P < 0.0001$) and positively correlated with both the number of nuclei per fiber ($r^2 = 0.60$, $n = 38$, $P < 0.0001$) and myonuclear domain ($r^2 = 0.49$, $n = 38$, $P < 0.0001$). When considering the effect of fiber diameter in analysis of covariance models, capillaries per fiber area did not vary with regards to phenotype but instead differed between treatments within phenotypes. Indeed, scrutinizing treatment values revealed that for a given diameter of muscle fiber, cold-acclimated birds that experienced the temperature drop were the only individuals expressing a significant change, with the number of capillaries per fiber area being 22% higher in this group than in their control counterparts (Fig. 4). However, this effect was weak since post hoc analysis could not detect significant differences among groups. The number of nuclei per millimeter of fiber differed both by phenotype and by treatment within phenotype when statistically controlling for the effect of fiber diameter (Table 2). For a given muscle fiber size, cold-acclimated birds had 11% more nuclei per millimeter of fiber compared with birds from the warm-transition phenotype. However, this effect was driven by cold-acclimated birds that had experienced the temperature drop, as post hoc analyses revealed no significant difference between birds from the warm-transition phenotype, which had comparable numbers of nuclei per millimeter to cold-acclimated control birds (Fig. 4). In contrast, cold-acclimated birds that had experienced the temperature change had 22% more nuclei per millimeter of fiber than their control counterparts (Fig. 4). Myonuclear domain showed the reverse pattern, also differing by phenotype and by treatment within phenotype when controlling for the effect of fiber diameter (Table 2, Fig. 4). Overall, muscle fibers from warm-transition birds had 13.8% larger amounts of cytoplasm per nuclei compared with cold-acclimated birds but, here again, the treatment effect was driven by cold-acclimated birds that had experienced the decline in temperature. In fact, those birds had a myonuclear domain 21.8% lower than their control counterparts. Myonuclear domain in these birds was also lower than that of all birds in the warm-transition phenotype (Fig. 4). Therefore, cold-acclimated birds maintained larger pectoralis muscle fibers than birds acclimated to warm conditions. For a given fiber size, these birds also rapidly increased the number of capillaries and the number of myonuclei when exposed to a sudden decrease in temperature, leading to a decline in myonuclear domain. In contrast, warm-acclimated birds experiencing the same increase in thermoregula-

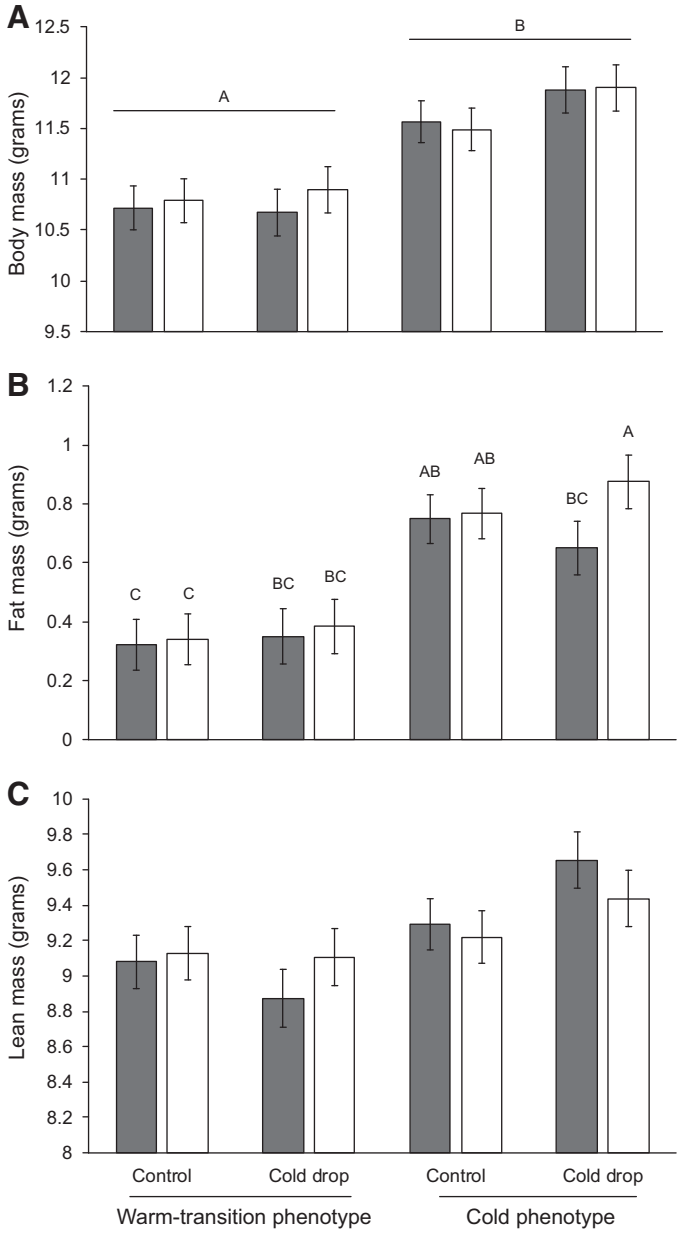

Fig. 3. Body composition before (shaded bars) and after (open bars) the acute temperature drop for the control and cold-drop groups within each phenotype. Total body mass (*A*), fat mass (*B*), and lean mass (*C*). Values are least square means ± SE, controlling for structural size and the random effect of bird ID. Bar with different letters presents statistically significant differences at $P < 0.05$.

tory demand showed no significant changes in muscle ultrastructure.

DISCUSSION

In this study, in addition to the expected higher food intake and larger fat reserves observed in birds maintaining a cold phenotype, we found that black-capped chickadees also maintained larger pectoralis muscle fiber diameters and, importantly, that cold-acclimated birds were able to rapidly recruit new myonuclei into their muscle fibers after exposure to a cold challenge, consequently decreasing myonuclear domain. Though myonuclear domains seem to be flexible (54), to our knowledge, this is the first time that such a rapid adjustment in muscle fiber ultrastructure is demonstrated during an acute thermal challenge. The rapid adjustments reported here are quite surprising, however, as previous studies suggest that several days are needed for birds to transition into a new phenotype with improved shivering heat production capacity (10, 30, 31). It is also important to emphasize here that these cellular changes were only visible in birds already acclimated to cold conditions that experienced the 15°C drop in temperature. Birds from the warm-transition group experiencing the same sudden decrease in temperature showed no such changes. These results strongly suggest, as expected, that chickadees maintaining a cold phenotype were better prepared to respond to a sudden decline in temperature, such as what could be experienced in their natural wintering environment.

Whole Organism

Cold-acclimated birds ate almost twice as much food (95% more, 90% when comparing only control groups) than individuals from the warm-transition phenotype. They were also 9% heavier and maintained twice as much fat as individuals kept at 20°C. Energy intake in cold-acclimated birds during the days preceding the experiment was 56.6 ± 1.36 kJ/day [taking into account digestive efficiency (8)]. This is only 14% lower than the daily energy expenditure (66 kJ/day) reported by Karasov et al. (17) for 5 wintering black-capped chickadees measured in the wild and living at −10°C (range −15 to 5°C). Therefore, our cold environment before the drop in temperature was as energy demanding, if not more so, than natural wintering conditions since our birds did not have to spend energy foraging for food.

Birds from both phenotypes changed their food consumption during the experiment, but not as expected. Individuals from the warm-transition phenotype reduced their intake during the period coinciding with the temperature drop, whether they experienced it or not, and increased their consumption afterward. In contrast, cold-acclimated birds increased food consumption in both experimental treatments. This was most apparent in those experiencing the decline in temperature, which had already increased their intake by 50% during the change and by 80% in the next 3 h compared with the

Table 2. *Summary of statistical results for the effect of phenotype and treatment on histological measures of black-capped chickadees*

		Model Statistics		
Measure	Factor	df	*F*	*P*
Fiber diameter	Phenotype	1	19.1	0.0001
	Treatment[phenotype]	2	0.06	0.94
Capillaries per fiber area	Phenotype	1	1.5	0.225
	Treatment[phenotype]	2	3.3	0.048
	Fiber diameter	1	22.0	<0.0001
Number of nuclei per millimeter of fiber	Phenotype	1	5.4	0.026
	Treatment[phenotype]	2	8.4	0.001
	Fiber diameter	1	32.0	<0.0001
Myonuclear domain	Phenotype	1	6.3	0.017
	Treatment[phenotype]	2	7.8	0.002
	Fiber diameter	1	54.6	<0.0001

df, degrees of freedom.

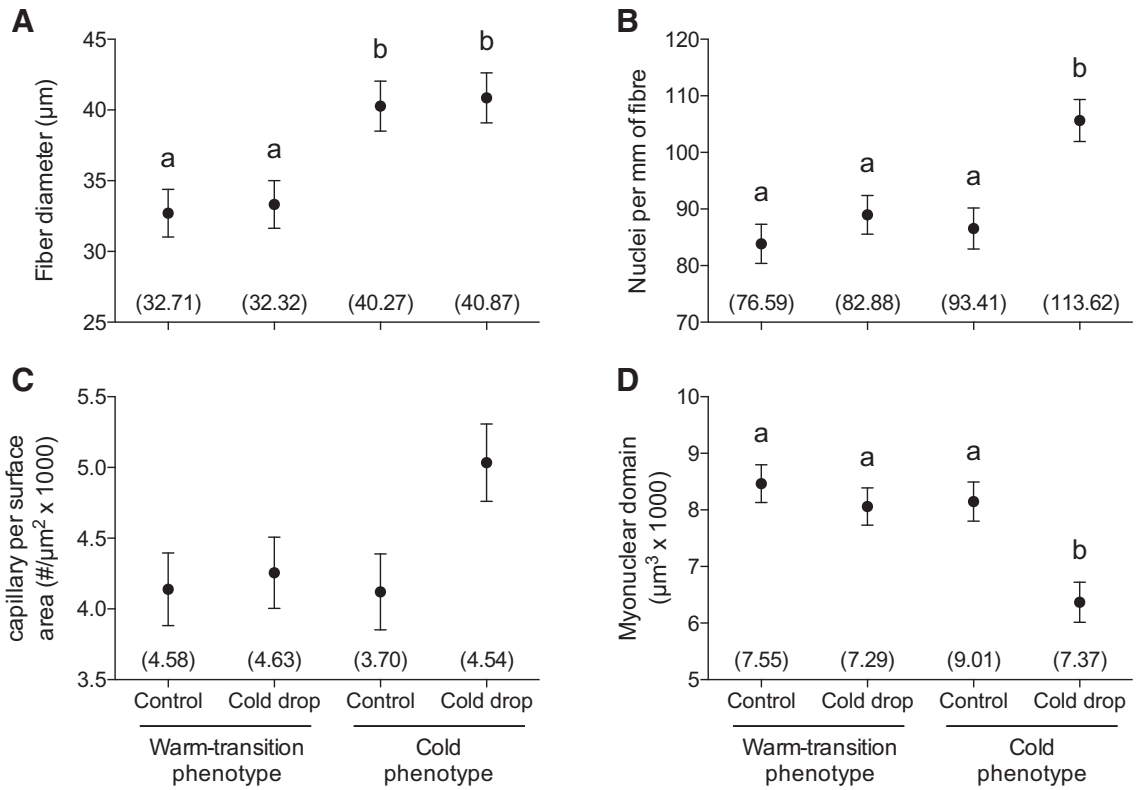

Fig. 4. Muscle histology metrics in the control and cold-drop groups within each phenotype. Fiber diameter size (*A*), number of nuclei per millimeter of fiber (*B*), capillaries per fiber area (*C*), and myonuclear domain (*D*). Values in *B*, *C*, and *D* are least square means ± SE controlling for the effect of fiber diameter. Numbers in parentheses represent raw, unadjusted mean values (see MATERIALS AND METHODS). Symbols with different letters present statistically significant differences at $P < 0.05$.

pre-experiment level. Therefore, birds from both treatments in each phenotype changed their food consumption in similar ways, independently from the effect of temperature change.

These findings are unexpected and suggest that food consumption patterns in captive chickadees are sensitive to disturbances (9, 31) since, to collect food intake data, we had to go in the rooms and change the cage trays at each of the three time periods. This seemingly benign disturbance (birds fed this way for the preceding 18 days) was apparently enough for warm-transition birds to reduce their food consumption momentarily, although individuals also experiencing the temperature drop, thus combining a second level of disturbance, remained at 66% of their predrop value. Disturbance had a much different effect in birds maintaining a cold phenotype, however. By the end of the experiment, all these individuals had increased their food consumption considerably, with a faster response in individuals experiencing both food-measurement and temperature-drop disturbances. These observations therefore suggest that the reaction to disturbance depends on phenotype, and most likely, on daily energy demand since birds having to support higher demands in the cold consumed more food, opposite to warm-transition birds. Presumably, this is a response to the perceived risk associated with the current state since a negative energy budget in the cold likely has a much greater impact on chances of survival than a negative budget at milder temperatures. It is expected that warm-transition birds readjusting to the new temperature after a cold drop would have increased their food consumption since intake rate generally correlates with energy consumption, but this may not be immediate at mild temperatures, preventing us from detecting changes in the first 3 h after temperature stabilization. Thus, in line with our predictions, cold-acclimated birds seemed better prepared to face unpredictable events and seemed able to respond quickly by increasing energy intake. These birds also presumably maintained a higher immediate spare capacity for food intake, as this was the only phenotype increasing food consumption [but note that McWilliams and Karasov (24) measured immediate spare capacity over 48 h]. Furthermore, cold-acclimated birds exposed to the temperature drop increased their intake rate most rapidly and were the only individuals gaining fat during the experiment. Devost et al. (9) reported a similar phenomenon in captive black-capped chickadees exposed to either a gradual increase or decrease in temperature. In both cases, birds increased their food consumption and changed their diet toward energy-rich items favoring high fattening rate.

Muscle Ultrastructure

After 21 days of stable conditions, cold-acclimated chickadees maintained 23% larger fiber diameters in their pectoralis muscles than warm-transition birds. This observation concurs with findings by Jimenez et al. (15), who reported larger pectoralis fibers in chickadees captured in the spring (April to May), thus just coming out of winter, than in individuals measured in summer (July to August) or in the fall (October to November). Muscle fiber diameter is highly correlated with fiber cross-sectional area (all birds in this study $r^2 = 0.97$, $n = 38$, $P < 0.0001$), and given that increasing fiber cross-sectional

area leads to higher contraction force and presumably increased heat production, maintaining large fiber diameters could be beneficial to the cold phenotype in chickadees. One clear advantage of large fibers is their lower basal metabolic rate, which results from the proportionally reduced energy cost of running the Na^+-K^+-ATPase for maintaining membrane potential (13). Investigations of the effect of body components (organ mass) on whole animal basal metabolic rate have reported a significant and predominant contribution of pectoralis or all skeletal muscles on this measure of physiological maintenance cost in birds (6, 25, 31, 57). Therefore, for small passerines in a situation in which a cold environment requires high daily energy expenditure (17, 44) and improved thermogenic capacity (29, 30), maintaining large-diameter muscle fibers could be a good strategy to improve heat production while minimizing maintenance energy demand (47).

Recent experimental evidence (2, 25, 42, 46, 52) showed that passerine birds can improve maximal shivering heat production without enlarging overall muscle size. For example, Milbergue, Blier, and Vézina (25) found a 20% higher summit metabolic rate in black-capped chickadees acclimated to −10°C compared with individuals kept at thermoneutrality (27°C), despite birds showing no significant mass difference in lean dry pectoralis or all other (carcass) muscles (see also Ref. 46). Therefore, cold acclimation in small birds seem to involve significant molecular-level regulation. Although the exact mechanisms have still to be described, several processes appear to be good candidates. For instance, after 6 wk of cold acclimation, dark-eyed juncos showed an increase in M_{sum} and transcription of genes promoting muscle growth and repair, despite a lack of change in pectoralis mass (42, 46). Myostatin, a muscle growth inhibitor, seems to regulate muscle growth patterns (19), and colder temperatures can downregulate the concentrations of myostatin, which in turn seems to allow pectoralis muscles to increase in size and mass in house sparrows (50). However, it should be noted here that myostatin expression did not change between winter and summer black-capped chickadees (46), though others have noted a change during temperature acclimation (7). Another potential molecular mechanism for regulating muscle size is the insulin-like growth factor (IGF) 1 pathway. Increases in IGF-1 concentrations have been linked to hypertrophic muscle growth (43), protein synthesis and increases in satellite cell proliferation (36), all patterns that we have seen in our study.

In contrast with warm-transition birds that expressed no changes, cold-acclimated chickadees showed a remarkably fast response in muscle ultrastructure when exposed to a temperature drop. Indeed, 3 h after temperature stabilization, these birds had more capillaries per fiber area and had 22% more nuclei per millimeter of fiber, and myonuclear domain was, correspondingly, 22% smaller in these fibers. Satellite cells are the seemingly finite stem-like population of myotube precursors that exist in close association with myotubes. They are recruited into the existing muscle fiber in times of strain or repair (3, 5, 41), and, as muscle tissue is a syncytium, satellite cells could be recruited into adjacent fibers to increase muscle fiber diameter (54). As more nuclei are recruited, myonuclear domain is then reduced, and each nucleus becomes responsible for servicing a smaller amount of cytoplasm (3). The recruitment of satellite cells into existing fibers is considered a rapid process, with activation and proliferation known to occur within 18 h of differentiation signaling in mice (61). However, cold-acclimated chickadees examined only 3 h after a 15°C drop in temperature already had 22% more nuclei per millimeter of muscle fibers in their pectoralis muscle, which is, to our knowledge, the fastest response reported yet.

A decrease in myonuclear domain during an acute thermal challenge has several implications. First, this change can be viewed as a decrease in protein-turnover load per nuclei if all nuclei are equally producing products, thus, decreasing the rate at which each nucleus has to operate. It could also imply that nuclei are not all producing products at rates fast enough for the needs, and thus recruiting more nuclei into existing fibers would allow for increasing protein-turnover rates (3). Second, the observed decrease in myonuclear domain may ultimately have consequences for fiber diameters. Others have found that exercise training causes a decrease in myonuclear domain, which is the precursor to increases in muscle fiber diameters (5). Therefore, it may be that the rapid cold-triggered addition of new nuclei into muscle fibers of cold-phenotype chickadees occurs before an even further increase in fiber diameter, which could hypothetically improve thermogenic capacity through higher force production.

To our knowledge such a rapid response in muscle ultrastructure has not been reported before in vertebrates. As this was visible only in cold-acclimated birds, it thus seems to us that this phenotype comes with some level of reserve capacity, taking the form of an ability to rapidly adjust muscle ultrastructure. It should be stated here, however, that this may also come at a cost since populations of muscle satellite cells may be finite (3). This could therefore mean that chickadees quickly recruiting satellite cells into muscle fibers as a means of safeguarding against exposure to sudden cold may be limited in their future muscle phenotypic adjustments, including muscle repair following intense activity.

The changes in muscle cells we observed here are clearly happening faster than adjustments in maximal thermogenic capacity (measured as M_{sum}). For example, Dubois, Hallot, and Vézina (10) tracked changes in M_{sum} in 3 passerine bird species, including black-capped chickadees, over 8 days after a 15°C drop or an 18°C increase in temperature, starting from a stable phenotype at 10°C. Of the three species, only white-throated sparrows showed the expected pattern (increase in the cold and decrease in the heat), but, even so, M_{sum} was only 4% higher after 8 days in the cold and did not differ significantly from before the change in temperature. Therefore, changes in whole animal thermogenic capacity were happening but clearly not within hours of a thermal challenge as seen here at the cell level. In fact, although temperature is a known driving force of M_{sum} variation (e.g., 41, 48, 51, 52, 55, 62), little is known of the rate at which birds can adjust their muscle phenotype and of what changes are required to improve maximal thermogenic capacity (but see Refs. 7, 42, 47, 63, and 64). What is known, however, is that in the wild, black-capped chickadees begin to improve their shivering heat production several months before the winter peak of cold (30, 49). These birds also maintain reserve capacity in M_{sum} at the coldest time of the year (32), presumably because this parameter is too slow to respond to day-to-day variation in temperature (10, 30, 49). Therefore, although changes in muscle fibers may happen within hours after a temperature drop, these could potentially only be a precursor of cellular adjustments ultimately leading to im-

proved shivering heat production at a later stage, which may or may not lead to an increase in muscle size (25, 31, 32), a phenomenon possibly taking days to weeks.

Improving thermogenic capacity is beneficial for survival in black-capped chickadees (18, 29) and seems to require preparation ahead of time (30, 32). As is typical at northern latitudes in the fall, these birds experience a gradual decline in ambient temperature each year. This likely acts as a signal, slowly increasing in intensity, that may be the trigger for changes in muscle cells (42, 46, 63, 64), development of capillaries (41, this study) and for changes in other tissues (20, 31). This would ultimately lead to the slow increase in thermogenic capacity reported in the wild (30, 31). In addition, maintaining a cold-acclimatized phenotype able to react quickly at the cell level to sudden, but relatively rare, drops in ambient temperature may be key to survival.

Perspective and Significance

The growing number of studies on phenotypic flexibility reporting trait amplitude variation and patterns are of great help in understanding how animals adjust their physiology to a changing environment (see Refs. 22, 23, 35, and 59 for reviews). However, the rate at which specific traits can change and whether this is critically limiting to organisms facing phenotypic mismatches has received much less attention. Knowledge is also lacking on how initial phenotype may help or limit adjustments to new constraints. Our study aimed at improving our understanding of this specific aspect of phenotypic flexibility by studying short-term responses to a sudden increase in thermoregulatory demand in two contrasted phenotypes of black-capped chickadees. We showed that major adjustments were rapidly happening in muscle ultrastructure but that this was only visible in birds already acclimated to cold, suggesting a certain level of spare capacity in muscle functions of birds maintaining a cold-acclimated phenotype. This finding is logical in a context in which birds living in cold environments may have very little time to respond to sudden increases in thermoregulatory demands before chances of survival begin to decline. Further experiments tracking phenotypic changes until stability are required to shed light on long-term consequences of phenotypic mismatches.

ACKNOWLEDGMENTS

We acknowledge Sarah Senécal and Justine Drolet for help in catching and caring for birds and assisting during an early test of the experiment. We are also grateful to Dr. Jason Meyers for help using confocal microscopy.

Current address of E. Cornelius Ruhs: Global and Planetary Health, Univ. of South Florida, Tampa, FL.

GRANTS

This research was supported by National Sciences and Engineering Research Council Discovery Grants to F. Vézina and O. P. Love; a Colgate University Research Council Grant to A. G. Jimenez; and Canada Foundation for Innovation (CFI) Awards to F. Vézina and O. P. Love; and Canada Research Chair funding to O. P. Love. E. Cornelius Ruhs was supported by a Fonds de recherche du Québec-Nature et technologies (FRQNT) Merit Scholarship Program for Foreign Students (PBEEE) Short-Term Research Fellowship. E. S. O'Connor received support from Colgate University's Office of Undergraduate Research.

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

F.V. and A.G.J. conceived and designed research; F.V., E.C.R., E.S.O., A.L.P., L.R., and A.G.J. performed experiments; F.V., E.C.R., and A.G.J. analyzed data; F.V., E.C.R., and A.G.J. interpreted results of experiments; F.V. and E.C.R. prepared figures; F.V. and E.C.R. drafted manuscript; F.V., E.C.R., E.S.O., O.P.L., and A.G.J. edited and revised manuscript; F.V., E.C.R., E.S.O., O.P.L., and A.G.J. approved final version of manuscript.

REFERENCES

1. **Barceló G, Salinas J, Cavieres G, Canals M, Sabat P.** Thermal history can affect the short-term thermal acclimation of basal metabolic rate in the passerine *Zonotrichia capensis*. *J Therm Biol* 34: 415–419, 2009. doi:10.1016/j.jtherbio.2009.06.008.
2. **Barceló G, Love OP, Vézina F.** Uncoupling basal and summit metabolic rates in white-throated sparrows: digestive demand drives maintenance costs, but changes in muscle mass are not needed to improve thermogenic capacity. *Physiological and Biochemical Zoology: PBZ* 90: 153–165, 2017. doi:10.1086/689290.
3. **Brooks NE, Schuenke MD, Hikida RS.** Ageing influences myonuclear domain size differently in fast and slow skeletal muscle of rats. *Acta Physiol (Oxf)* 197: 55–63, 2009. doi:10.1111/j.1748-1716.2009.01983.x.
4. **Brown K, Jimenez AG, Whelan S, Lalla K, Hatch SA, Elliott KH.** Muscle fiber structure in an aging long-lived seabird, the black-legged kittiwake (*Rissa tridactyla*). *J Morphol* 280: 1061–1070, 2019. doi:10.1002/jmor.21001.
5. **Bruusgaard JC, Johansen IB, Egner IM, Rana ZA, Gundersen K.** Myonuclei acquired by overload exercise precede hypertrophy and are not lost on detraining. *Proc Natl Acad Sci USA* 107: 15111–15116, 2010. doi:10.1073/pnas.0913935107.
6. **Chappell MA, Bech C, Buttemer WA.** The relationship of central and peripheral organ masses to aerobic performance variation in house sparrows. *J Exp Biol* 202: 2269–2279, 1999.
7. **Cheviron ZA, Swanson DL.** Comparative transcriptomics of seasonal phenotypic flexibility in two North American songbirds. *Integr Comp Biol* 57: 1040–1054, 2017. doi:10.1093/icb/icx118.
8. **Cornelius EA, Vézina F, Regimbald L, Hallot F, Petit M, Love OP, Karasov WH.** Chickadees faced with unpredictable food increase fat reserves but certain components of their immune function decline. *Physiol Biochem Zool* 90: 190–200, 2017. doi:10.1086/689913.
9. **Devost I, Hallot F, Milbergue M, Petit M, Vézina F.** Lipid metabolites as markers of fattening rate in a non-migratory passerine: effects of ambient temperature and individual variation. *Comp Biochem Physiol A Mol Integr Physiol* 177: 18–26, 2014. doi:10.1016/j.cbpa.2014.07.014.
10. **Dubois K, Hallot F, Vézina F.** Basal and maximal metabolic rates differ in their response to rapid temperature change among avian species. *J Comp Physiol B* 186: 919–935, 2016. doi:10.1007/s00360-016-1001-5.
11. **Guglielmo CG, McGuire LP, Gerson AR, Seewagen CL.** Simple, rapid, and non-invasive measurement of fat, lean, and total water masses of live birds using quantitative magnetic resonance. *J Ornithol* 152, *S1*: 75–85, 2011. doi:10.1007/s10336-011-0724-z.
12. **Hohtola E.** Shivering thermogenesis in birds and mammals. Twelfth International Hibernation Symposium. Life in the cold: Evolution, mechanisms, adaptation and application. Fairbanks Institute of Arctic Biology: University of Alaska, 2004.
13. **Jimenez AG, Dillaman RM, Kinsey ST.** Large fibre size in skeletal muscle is metabolically advantageous. *Nat Commun* 4: 2150, 2013. doi:10.1038/ncomms3150.
14. **Jimenez AG, Kinsey ST.** Nuclear DNA content variation associated with muscle fiber hypertrophic growth in fishes. *J Comp Physiol B* 182: 531–540, 2012. doi:10.1007/s00360-011-0635-6.
15. **Jimenez AG, O'Connor E, Brown K, Briggs C.** Seasonal muscle ultrastructure plasticity and resistance of muscle structural changes during temperature increases in resident black-capped chickadees and rock pigeons. *J Exp Biol* 222: jeb201855, 2019. doi:10.1242/jeb.201855.
16. **Jimenez AG, Williams JB.** Differences in muscle fiber size and associated energetic costs in phylogenetically paired tropical and temperate birds. *Physiol Biochem Zool* 87: 752–761, 2014. doi:10.1086/677922.
17. **Karasov WH, Brittingham MC, Temple SA.** Daily energy and expenditure by black-capped chickadees (*Parus atricapillus*) in winter. *Auk* 109: 393–395, 1992. doi:10.2307/4088213.
18. **Latimer CE, Cooper SJ, Karasov WH, Zuckerberg B.** Does habitat fragmentation promote climate-resilient phenotypes? *Oikos* 127: 1069–1080, 2018. doi:10.1111/oik.05111.

19. **Lee SJ, McPherron AC.** Regulation of myostatin activity and muscle growth. *Proc Natl Acad Sci USA* 98: 9306–9311, 2001. doi:10.1073/pnas.151270098.
20. **Liknes ET, Swanson DL.** Phenotypic flexibility of body composition associated with seasonal acclimatization in passerine birds. *J Therm Biol* 36: 363–370, 2011. doi:10.1016/j.jtherbio.2011.06.010.
21. **Mathieu-Costello O, Agey PJ, Quintana ES, Rousey K, Wu L, Bernstein MH.** Fiber capillarization and ultrastructure of pigeon pectoralis muscle after cold acclimation. *J Exp Biol* 20: 3211–3220, 1998.
22. **McKechnie AE.** Phenotypic flexibility in basal metabolic rate and the changing view of avian physiological diversity: a review. *J Comp Physiol B* 178: 235–247, 2008. doi:10.1007/s00360-007-0218-8.
23. **McKechnie AE, Noakes MJ, Smit B.** Global patterns of seasonal acclimatization in avian resting metabolic rates. *J Ornithol* 156, *Suppl* 1: 367–376, 2015. doi:10.1007/s10336-015-1186-5.
24. **McWilliams SR, Karasov WH.** Spare capacity and phenotypic flexibility in the digestive system of a migratory bird: defining the limits of animal design. *Proc Biol Sci* 281: 20140308, 2014. doi:10.1098/rspb.2014.0308.
25. **Milbergue MS, Blier PU, Vézina F.** Large muscles are beneficial but not required for improving thermogenic capacity in small birds. *Sci Rep* 8: 14009, 2018. doi:10.1038/s41598-018-32041-w.
26. **Nyack AC, Locke BR, Valencia A, Dillaman RM, Kinsey ST.** Scaling of postcontractile phosphocreatine recovery in fish white muscle: effect of intracellular diffusion. *Am J Physiol Regul Integr Comp Physiol* 292: R2077–R2088, 2007. doi:10.1152/ajpregu.00467.2006.
27. **Packard GC, Boardman TJ.** The misuse of ratios, indices, and percentages in ecophysiological research. *Physiol Zool* 61: 1–9, 1988. doi:10.1086/physzool.61.1.30163730.
28. **Packard GC, Boardman TJ.** The use of percentages and size-specific indices to normalize physiological data for variation in body size: wasted time, wasted effort? *Comp Biochem Physiol A Mol Integr Physiol* 122: 37–44, 1999. doi:10.1016/S1095-6433(98)10170-8.
29. **Petit M, Clavijo-Baquet S, Vézina F.** Increasing winter maximal metabolic rate improves intrawinter survival in small birds. *Physiol Biochem Zool* 90: 166–177, 2017. doi:10.1086/689274.
30. **Petit M, Lewden A, Vézina F.** Intra-seasonal flexibility in avian metabolic performance highlights the uncoupling of basal metabolic rate and thermogenic capacity. *PLoS One* 8: e68292, 2013. doi:10.1371/journal.pone.0068292.
31. **Petit M, Vézina F.** Phenotype manipulations confirm the role of pectoral muscles and haematocrit in avian maximal thermogenic capacity. *J Exp Biol* 217: 824–830, 2014. doi:10.1242/jeb.095703.
32. **Petit M, Vézina F.** Reaction norms in natural conditions: how does metabolic performance respond to weather variations in a small endotherm facing cold environments? *PLoS One* 9: e113617, 2014. doi:10.1371/journal.pone.0113617.
33. **Piersma T.** Energetic bottlenecks and other design constraints in avian annual cycles. *Integr Comp Biol* 42: 51–67, 2002. doi:10.1093/icb/42.1.51.
34. **Piersma T, Drent J.** Phenotypic flexibility and the evolution of organismal design. *Trends Ecol Evol* 18: 228–233, 2003. doi:10.1016/S0169-5347(03)00036-3.
35. **Piersma T, van Gils JA.** *The Flexible Phenotype: A Body-Centred Integration of Ecology, Physiology, and Behaviour*. Oxford, UK: Oxford University Press, 2011.
36. **Qaisar R, Larsson L.** What determines myonuclear domain size? *Indian J Physiol Pharmacol* 58: 1–12, 2014.
38. **Rising JD, Somers KM.** The measurement of overall body size in birds. *Auk* 106: 666–674, 1989.
39. **Ross TT, Overton JD, Houmard KF, Kinsey ST.** β-GPA treatment leads to elevated basal metabolic rate and enhanced hypoxic exercise tolerance in mice. *Physiol Rep* 5: e13192, 2017. doi:10.14814/phy2.13192.
40. **Schmalbruch H, Hellhammer U.** The number of nuclei in adult rat muscles with special reference to satellite cells. *Anat Rec* 189: 169–175, 1977. doi:10.1002/ar.1091890204.
41. **Sinha-Hikim I, Roth SM, Lee MI, Bhasin S.** Testosterone-induced muscle hypertrophy is associated with an increase in satellite cell number in healthy, young men. *Am J Physiol Endocrinol Metab* 285: E197–E205, 2003. doi:10.1152/ajpendo.00370.2002.
42. **Stager M, Swanson DL, Cheviron ZA.** Regulatory mechanisms of metabolic flexibility in the dark-eyed junco (Junco hyemalis). *J Exp Biol* 218: 767–777, 2015. doi:10.1242/jeb.113472.
43. **Stitt TN, Drujan D, Clarke BA, Panaro F, Timofeyva Y, Kline WO, Gonzalez M, Yancopoulos GD, Glass DJ.** The IGF-1/PI3K/Akt pathway prevents expression of muscle atrophy-induced ubiquitin ligases by inhibiting FOXO transcription factors. *Mol Cell* 14: 395–403, 2004. doi:10.1016/S1097-2765(04)00211-4.
44. **Swanson DL.** *Seasonal metabolic variation in birds: functional and mechanistic correlates*. In: *Current Ornithology*, edited by Thompson CF. Basel, Switzerland: Springer, 2010, vol. 17, p. 75–129. doi:10.1007/978-1-4419-6421-2_3.
46. **Swanson DL, King MO, Harmon E.** Seasonal variation in pectoralis muscle and heart myostatin and tolloid-like proteinases in small birds: a regulatory role for seasonal phenotypic flexibility? *J Comp Physiol B* 184: 249–258, 2014. doi:10.1007/s00360-013-0798-4.
47. **Swanson DL, McKechnie AE, Vézina F.** How low can you go? An adaptive energetic framework for interpreting basal metabolic rate variation in endotherms. *J Comp Physiol B* 187: 1039–1056, 2017. doi:10.1007/s00360-017-1096-3.
48. **Swanson DL, Merkord C.** Seasonal phenotypic flexibility of flight muscle size in small birds: a comparison of ultrasonography and tissue mass measurements. *J Ornithol* 154: 119–127, 2013. doi:10.1007/s10336-012-0877-4.
49. **Swanson DL, Olmstead KL.** Evidence for a proximate influence of winter temperature on metabolism in passerine birds. *Physiol Biochem Zool* 72: 566–575, 1999. doi:10.1086/316696.
50. **Swanson DL, Sabirzhanov B, Vandezande A, Clark TG.** Seasonal variation of myostatin gene expression in pectoralis muscle of house sparrows (*Passer domesticus*) is consistent with a role in regulating thermogenic capacity and cold tolerance. *Physiological and Biochemical Zoology: PBZ* 82: 121–128, 2009. doi:10.1086/591099.
51. **Swanson DL, Vézina F.** Environmental, ecological and mechanistic drivers of avian seasonal metabolic flexibility in response to cold winters. *J Ornithol* 156, *Suppl* 1: 377–388, 2015. doi:10.1007/s10336-015-1192-7.
52. **Swanson DL, Zhang Y, King M.** Mechanistic drivers of flexibility in summit metabolic rates of small birds. *PLoS One* 9: e101577, 2014. doi:10.1371/journal.pone.0101577.
54. **Van der Meer SFT, Jaspers RT, Degens H.** Is the myonuclear domain size fixed? *J Musculoskelet Neuronal Interact* 11: 286–297, 2011.
55. **Vézina F, Dekinga A, Piersma T.** Phenotypic compromise in the face of conflicting ecological demands: an example in red knots Calidris canutus. *J Avian Biol* 41: 88–93, 2010. doi:10.1111/j.1600-048X.2009.04763.x.
56. **Vézina F, Dekinga A, Piersma T.** Shorebirds' seasonal adjustments in thermogenic capacity are reflected by changes in body mass: how preprogrammed and instantaneous acclimation work together. *Integr Comp Biol* 51: 394–408, 2011. doi:10.1093/icb/icr044.
57. **Vézina F, Gerson AR, Guglielmo CG, Piersma T.** The performing animal: causes and consequences of body remodeling and metabolic adjustments in red knots facing contrasting thermal environments. *Am J Physiol Regul Integr Comp Physiol* 313: R120–R131, 2017. doi:10.1152/ajpregu.00453.2016.
58. **Vézina F, Jalvingh KM, Dekinga A, Piersma T.** Thermogenic side effects to migratory predisposition in shorebirds. *Am J Physiol Regul Integr Comp Physiol* 292: R1287–R1297, 2007. doi:10.1152/ajpregu.00683.2006.
59. **Vézina F, Salvante KG.** Behavioral and physiological flexibility are used by birds to manage energy and support investment in the early stages of reproduction. *Curr Zool* 56: 767–792, 2010. doi:10.1093/czoolo/56.6.767.
60. **Wright NA, Alison M.** *The Biology of Epithelial Cell Populations*. Oxford, UK: Oxford University Press, 1984, vol 1.
61. **Zammitt PS.** Kinetics of myoblast proliferation show that resident satellite cells are competent to fully regenerate skeletal muscle fibers. *Exp Cell Res* 281: 39–49, 2002. doi:10.1006/excr.2002.5653.
62. **Zhang Y, Eyster K, Swanson DL.** Context-dependent regulation of pectoralismyostatin and lipid transporters by temperature and photoperiod in dark-eyed juncos. *Curr Zool* 64: 23–31, 2018. doi:10.1093/cz/zox020.
63. **Zhang Y, Eyster K, Liu JS, Swanson DL.** 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). *J Exp Biol* 218: 2190–2200, 2015. doi:10.1242/jeb.121822.
64. **Zhang Y, King MO, Harmon E, Swanson DL.** Summer-to-winter phenotypic flexibility of fatty acid transport and catabolism in skeletal muscle and heart of small birds. *Physiol Biochem Zool* 88: 535–549, 2015. doi:10.1086/682154.