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RESEARCH ARTICLE

Seasonal muscle ultrastructure plasticity and resistance of muscle structural changes during temperature increases in resident black-capped chickadees and rock pigeons

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ABSTRACT

Resident birds in temperate zones respond to seasonally fluctuating temperatures by adjusting their physiology, such as changes in basal metabolic rate or peak metabolic rate during cold exposure, or altering their organ sizes, so as to match the thermogenic requirements of their current environment. Climate change is predicted to cause increases in the frequency of heat and cold wave events, which could increase the likelihood that birds will face an environmental mismatch. Here, we examined seasonality and the effects of acute and chronic heat shock to 33°C and subsequent recovery from heat shock on the ultrastructure of the superficial pectoralis muscle fiber diameter, myonuclear domain (MND) and capillary density in two temperate bird species of differing body mass, the black-capped chickadee (*Poecile atricapillus*) and the rock pigeon (*Columba livia*). We found that muscle fiber ultrastructure did not change with heat treatment. However, in black-capped chickadees, there was a significant increase in fiber diameter in spring phenotype birds compared with summer phenotype birds. In rock pigeons, we saw no differences in fiber diameter across seasons. Capillary density did not change as a function of fiber diameter in black-capped chickadees, but did change seasonally, as did MND. Across seasons, as fiber diameter decreased, capillary density increased in the pectoralis muscle of rock pigeons. For both species in this study, we found that as fiber diameter increased, so did MND. Our findings imply that these two temperate birds employ different muscular growth strategies that may be metabolically beneficial to each.

KEY WORDS: Seasonality, Fiber diameter, Capillary density, Myonuclear domain, Temperate resident birds

INTRODUCTION

There is ample evidence that the Earth is warming, likely as a result of anthropogenic activity (Thompson, 2010). Environmental stresses brought about by climate change, particularly thermal stress, may play a key role in both setting biogeographic boundaries (Pudalov et al., 2017; Zaifman et al., 2017) and driving evolutionary patterns on broad spatial and temporal scales (Chown et al., 2010). Birds breeding in temperate zones where seasonal temperatures drastically fluctuate respond to these changes by either migrating to regions with more agreeable temperatures or altering their phenotype to deal with changing conditions (Swanson, 2010). Seasonal changes in temperature are a major factor for seasonal adjustments in physiology, such as basal metabolic rate (BMR) or peak metabolic rate during cold exposure ($\dot{M}_{sum}$) (Swanson, 2010). Therefore, birds have phenotypic flexibility, or a seasonal phenotype that aligns with the thermogenic requirements of their current environment (Swanson, 2010). We would assume that predicted increases in the frequency of heat and cold wave events due to climate change (Jentsch et al., 2007) would more frequently place individuals in an environment where their phenotype does not match the current conditions (Nussey et al., 2005).

As part of avian phenotypic plasticity across seasons, the size and composition of an animal's organs can be altered, which may have implications for whole-animal traits such as BMR (Piersma and Lindström, 1997). Changes in pectoralis muscle mass seem to be common during pre-migration. For example, one study quantified muscle fiber hypertrophy in three species of shore birds in winter and in the pre-migratory phenotype (Evans et al., 1992), and in two of the three species there was a concomitant increase in mitochondrial concentration as muscle fiber diameter increased. Eared grebes also demonstrated hypertrophic muscle growth with increases in aerobic capacity prior to the autumn migration (Gaunt et al., 1990; Piersma and Lindström, 1997). An ultrastructural study in catbirds (*Dumetella carolinensis*) found that muscle mass increased via hypertrophy in migrating birds; however, aerobic capacity remained constant (Marsh, 1984). Alteration of morphology of the pectoralis muscle is a common tactic employed by many bird species that occupy temperate regions to better match the energy demands during changing seasons (Swanson, 2010; Piersma and van Gils, 2011). Pectoralis muscle size alterations are especially pronounced between summer and winter seasons (Swanson, 1991; O'Connor, 1995; Cooper, 2002), though American goldfinches show no changes in pectoralis muscle morphology (Carey et al., 1978). Winter acclimatization in small passerine birds is frequently marked by increases in $\dot{M}_{sum}$, and by increased thermogenic capacity, which is often correlated to 'pectoralis muscle hypertrophy' (Cooper, 2002; O'Connor, 1995; Swanson, 2010). For example, in house sparrows (*Passer domesticus*), winter phenotype birds increased their pectoralis muscle size, which was linked to increases in peak metabolic rate potentially for increased thermoregulation during winter (Swanson, 2010). Many of these increases in pectoralis muscle mass are described as hypertrophic growth (e.g. Swanson, 1991; O'Connor, 1995; Cooper, 2002); however, whether this occurs as a result of hypertrophy (increases in muscle mass due to increases in muscle fiber diameter) or hyperplasia (increases in muscle mass due to increases in fiber number) (Swanson, 1991; O'Connor, 1995; Cooper, 2002; Kinsey et al., 2011) has yet to be determined in the case of seasonal phenotypic changes.

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The pectoralis muscle complex is the largest organ in the avian body, and is responsible for highly metabolically demanding processes such as flight and shivering (Greenwalt, 1962). Muscle fiber diameter in birds is constrained to a size range of 10–100 µm in diameter. These size limitations are likely a balance between diffusion and metabolic cost savings to the organism (Kinsey et al., 2011; Jimenez et al., 2013). That is, small fibers have short diffusion distances over which metabolically important molecules such as O_2 and ATP have to diffuse, promoting fast diffusion rates (Kinsey et al., 2011). In contrast, large fiber diameters are metabolically cheaper to maintain, thus potentially providing a strong selection pressure for larger muscle fibers (Jimenez et al., 2013). The balance between these two constraints on muscle fiber size is described by the 'optimal fiber hypothesis' (Johnston, 2006).

As muscle is a post-mitotic tissue, to grow hypertrophically, new nuclei must be drawn into the fiber itself from satellite cells, which are found in the basement membrane of each muscle fiber, and which are limited in number throughout the lifespan of an animal (Jimenez and Kinsey, 2012). The myonuclear domain (MND) is defined as the amount of cytoplasm within a muscle fiber that each nucleus is responsible for servicing (Qaisar and Larsson, 2014). The MND is an important physiological trait in muscle tissue. It is often the case that the MND is larger in larger muscle fibers, implying an increased workload per myonucleus. However, recruiting new nuclei into a myotube during hypertrophy may not necessarily cause an increase in MND. This would only happen if cell volume growth outpaces the recruitment of new nuclei during hypertrophy (Deschenes, 2004; Jimenez and Kinsey, 2012). Capillary density is also a key trait to measure during heat stress, as capillaries provide oxygen and nutrients to power muscles. Capillary density usually follows the aerobic potential around the muscle fiber, meaning that slow oxidative fibers have a higher density of capillaries than do fast glycolytic (anaerobic) ones (Torrella et al., 1998).

While many physiological responses to heat stress have been thoroughly studied, not much is known about how muscle structure responds to heat stress or what, if any, ultrastructural changes happen in the pectoralis muscle in birds across seasons. Here, we measured cell-level muscle ultrastructural changes in two species of resident birds in central New York across three seasons. Specifically, during each season, we examined the effects of chronic and acute heat shock on the muscle fiber diameter, MND and capillary density in two temperate bird species of differing body mass and ecological strategies, the black-capped chickadee [*Poecile atricapillus* (Linnaeus 1766)] and the rock pigeon (*Columba livia* Gmelin 1789). Large body masses have low surface area to volume ratios and low thermal conductance; thus, thermogenic costs in larger birds should vary less across seasons compared with those in small birds (Swanson, 2010). At the cell level, seasonal variation in BMR in winter in birds over 200 g, like the rock pigeon, is less comparable to that in small birds with higher surface area:volume ratios, such as the black-capped chickadee (Weathers and Caccamise, 1975; but see McKechnie, 2008). We assumed that both of these species would demonstrate a muscle mass increase during colder months (Milbergue et al., 2018; Saarela and Hohtola, 2003); however, Swanson et al. (2014) did not find changes in pectoralis size from summer to winter in chickadees. We predicted that small birds with higher thermogenic requirements would have more drastic muscle ultrastructural changes compared with larger birds during the more thermally demanding seasons. To our knowledge, this is the first study to look at ultrastructural changes in pectoralis muscle during seasonal acclimatization in two resident temperate birds of differing body mass.

MATERIALS AND METHODS

Animal collection

We caught autumn phenotype birds in October–November 2017 (average monthly temperature, 16°C); spring phenotype birds in April–May 2018 (average monthly temperature, 15°C); and summer phenotype birds in July–August 2018 (average monthly temperature, 28°C). Black-capped chickadees were caught in Madison County, NY, USA, using mist-nets and potter traps and were then transported to Colgate University in cages. Rock pigeons were also caught in Madison County, NY, USA, at night-time by hand and transported back to Colgate University in cages. Each bird was caged individually. None of our collected birds were actively molting. Birds were randomly divided into treatment groups as described below (Table 1). Birds were provided with water and food *ad libitum* during their time in captivity, and levels were checked three times per day. Daylight in each environmental chamber matched the daylight length of each season. All birds were caught within 60 km of Colgate University. All treatments described below were approved by Colgate University's Animal Care and Use Committee (IACUC).

Treatment groups

For each seasonal phenotype, each species of bird was further divided into an acute treatment group and a 5 day chronic treatment group. Within acute and chronic treatments, birds were further randomly assigned to a control, heat shock treatment (33°C) or recovery from heat shock treatment group. Control acute treatment birds were held at 22°C for 9 h after collection, acutely heat-shocked birds were placed in a Percival chamber with an IntellusUltra control system (CTH-7272) and held at 33°C for 9 h, and acute recovery birds were also heat shocked for 9 h, but were then allowed to recover from heat shock overnight (9–10 h) at 22°C. Control chronic treatment birds were held at 22°C for 5 days after collection, heat-shocked chronic treatment birds were heat shocked to 33°C for 6 h each day for 5 days, and recovery chronic treatment birds were also heat shocked, but then allowed to recover after the day 5 heat shock for 9–10 h at 22°C (Fig. 1). However, we were not aiming to test maximal increases in temperature, which, it could be argued, these animals may never see in the wild or in the near future. Therefore, we used 33°C as an ecologically relevant temperature for the central New York area, and one that has been common in recent years across all

Table 1. Sample size and sampling period for collection of black-capped chickadees and rock pigeons in central New York

			Acute (*N*)		Chronic (*N*)	
Season	Period		BC	RP	BC	RP
Autumn	Oct.–Nov.	Control	4	5	3	4
		Heat shock	4	6	5	4
		Recovery	3	5	4	3
Spring	Apr.–May	Control	3	3	3	4
		Heat shock	3	3	3	3
		Recovery	3	3	2	3
Summer	Jul.–Aug.	Control	2	3	2	3
		Heat shock	3	3	3	3
		Recovery	3	3	3	3

BC, black-capped chickadees; RP, rock pigeons; *N*, number of individuals.

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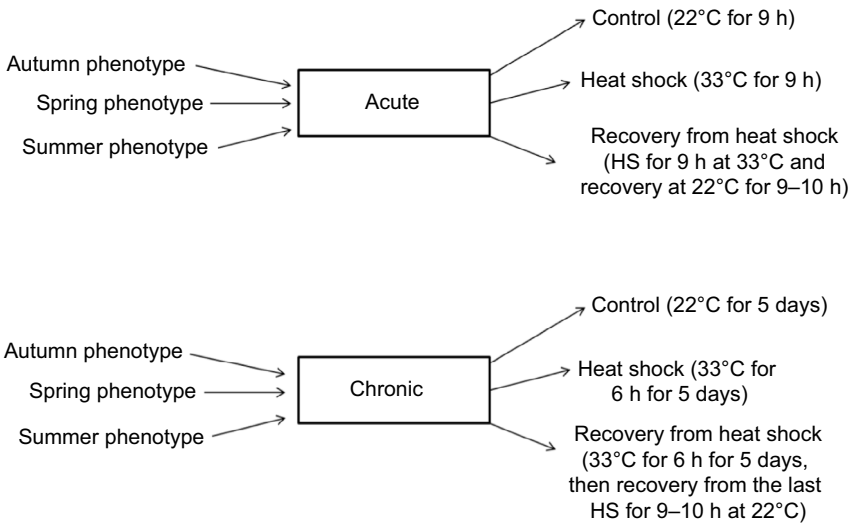

Fig. 1. Schematic depiction of the experimental treatments. Acute and chronic treatments for black-capped chickadee and rock pigeon for each of the three seasons are shown. HS, heat shock. Sample sizes are listed in Table 1.

three seasons measured, even though 33°C may be within the thermal neutral zone of both of these species, depending on phenotype. Birds were heat shocked from 09:00 h to 18:00 h for acute treatments and 09:00 h to 15:00 h for chronic treatments. Birds were killed after each of their assigned treatments using CO_2, and superficial pectoralis muscle was collected and placed in fixative (see below). Sample sizes for each group are given in Table 1.

Muscle sectioning and staining

After fixing pectoralis muscle in 4% paraformaldehyde, we placed muscle sections in 30% sucrose overnight to cryoprotect samples. Tissues were mounted in optimal cutting-temperature (OCT) compound and allowed to equilibrate to −20°C in a Microm HM 505N cryostat before sectioning. Sections were cut at 30 µm, picked up on plus slides, air-dried at room temperature, and then stained with a 250 mg ml^{-1} solution of wheatgerm agglutinin (WGA) with Alexa Fluor 488, 4′,6-diamidino-2-phenylindole (DAPI) and *Griffonia simplicifolia* lectin (GSL) 694 for 30 min, then rinsed in avian Ringer's solution for 60 min. WGA is a lectin that binds to glycoproteins on the basement membrane of the fiber sarcolemma and effectively outlines the fiber periphery to allow measurement of fiber size (Wright, 1984; Nyack et al., 2007). DAPI binds to nuclei and GSL 694 binds to capillaries. Stained slides were examined with a Zeiss 710 laser filter confocal microscope. Polygons were traced along the fiber periphery using ImageJ (Nyack et al., 2007; Jimenez et al., 2013). Each stain was analyzed individually per image. Polygons were initially drawn in WGA images in ImageJ 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. If there were oblique sections, we did not use that section to quantify any of our parameters. We did not count any fibers that showed any degree of muscle fascicle separation or freeze fracture. Forty-five fibers were randomly chosen and measured to obtain averages for each individual black-capped chickadee. Rock pigeon pectoralis muscle was composed of two populations of fibers, a small aerobic fiber population (small fibers) and a large, likely glycolytic muscle fiber population (large fibers) (Grinyer and George, 1969; Viscor et al., 1992). We separated each of these fiber types and counted 45 fibers of the small fibers and up to 30 of the large fibers per individual.

Nuclear domain determination

Fiber margins were traced using ImageJ and resultant polygons were used to calculate fiber cross-sectional area, while nuclear cross-sectional area and diameter (from fiber cross-sections) and nuclear length (from longitudinal sections) were calculated by outlining DAPI-stained nuclei. The number of nuclei per millimeter of fiber (X) was calculated following the method of Schmalbruch and Hellhammer (1977):

$$X = NL/d + l, \quad (1)$$

where N represents the number of myonuclei per cross-section of fiber, L is the desired length of the fiber segment (1000 mm), d is the thickness of the section (30 µm) and l is the mean length of a muscle nucleus. The volume of cytoplasm per nucleus or MND (Y) was calculated as:

$$Y = CL/X, \quad (2)$$

where C is the cross-sectional area of the muscle fiber measured for each species. Nuclear number volume, which is the number of nuclei per volume of cell, is the inverse of the MND, Y.

Capillary density

Fiber cross-sectional area, number of capillaries around the fiber and capillary density were analyzed using ImageJ. Capillary counts were measured by manual counting of the number of capillaries touching the fiber. Capillary density was obtained by dividing the capillary counts by muscle fiber cross-sectional area of each individual fiber (Ross et al., 2017).

Statistics

We used an ANOVA to examine the influence of season on fiber diameter for black-capped chickadees. We used a multiway ANOVA for rock pigeons to also assess the influence of fiber size (i.e. large versus small muscle fibers), season and the interaction between those factors on fiber diameter. In addition, we examined differences in mass between seasons for each species using ANOVA. We used Tukey's honest significant difference test as a *post hoc* test to examine effects of different levels of each significant factor.

To assess correlations within each species, we used Pearson correlation coefficients between each variable. We assessed the significance of pairwise comparisons of each variable and used Holm's method to correct P-values to account for multiple comparisons.

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We used Shapiro–Wilk tests to assess normality of the data and Bartlett's test to assess equal variance between groups for variables collected. Because data were not normally distributed and most variables did not have equal variance among groups, we examined univariate relationships between each variable and treatment as well as each variable and phenotype using Kruskal–Wallis tests. We used Dunn's test as a *post hoc* analysis of each significant response variable to examine differences between group levels as implemented in the FSA package (Ogle et al., 2018) using a corrected α. For each species, we used fiber diameter, capillary density, number of nuclei and MND as predictor variables to describe muscle histology. We visualized differences between groups by *z*-transforming data to a mean=0 and s.d.=1.

For all tests, we set α=0.05 to assume statistical significance. All data are presented as means±s.e.m., and data were visualized using ggplot2 (Wickham, 2016). All analyses were conducted in R 3.5.2 (https://www.R-project.org/).

RESULTS

There were significant correlations between all pairwise comparisons except number of nuclei and MND for rock pigeons (Table 2; Fig. S1). In contrast, for the black-capped chickadee muscle histology, only number of nuclei and fiber diameter showed significant pairwise correlations (Table 2; Fig. S1).

In black-capped chickadees, experimental treatments were not related to muscle fiber diameter, MND, number of nuclei or capillary density (Table 3; Fig. S2). In contrast, fiber diameter, MND, number of nuclei and capillary density were significantly different between seasonal phenotypes (Table 4, Fig. 2A), although not all seasons within a variable were significantly different (Table 5, Fig. 2A). In black-capped chickadees, average capillary density and MND increased and number of nuclei decreased between birds trapped in summer and autumn. In contrast black-capped chickadees trapped in summer had smaller fiber diameter and neither MND nor fiber diameter differed from those of individuals trapped in spring (Table 5, Fig. 2A). In addition, body mass did not change in birds trapped across seasons ($F_{2,49}$=0.03, P=0.98).

Similarly, in rock pigeons, experimental treatments were not related to muscle fiber diameter, MND, number of nuclei or capillary density (P>0.26 for all variables; Table 3; Fig. S3). Capillary density and number of nuclei were significantly different across phenotypes (P<0.05; Table 4, Fig. 2B). In rock pigeons, from autumn to spring, capillary density increased while the number of nuclei decreased. From autumn to summer, capillary density increased. From spring to summer, capillary density decreased (Table 6, Fig. 2B).

Fiber diameter varied across seasons in black-capped chickadees (F=3.65, P=0.03). Pairwise comparisons showed significant differences between data for the summer (31.4±1.1 µm) and spring (34.5±1.0 µm), and no differences between autumn (32.4±0.4 µm) and spring or summer (P>0.14; Fig. 3). In rock pigeons, fiber diameter did not change significantly between phenotypes (F=0.25, P=0.78), but did differ between fiber type/size (F=100.0, P<0.001), and the interaction between phenotype and fiber size (F=4.4, P=0.01; Fig. 3). In addition, body mass did not change across seasons ($F_{2,57}$=2.61, P=0.08).

Table 2. Pearson *r* correlation matrix for black-capped chickadees (above diagonal) and rock pigeons (below diagonal)

	No. of nuclei	Capillary density	MND	Fiber diameter
No. of nuclei	–	**−0.38**	**−0.77**	0.1
Capillary density	**−0.34**	–	0.23	0.12
MND	0.09	**−0.57**	–	**0.47**
Fiber diameter	**0.42**	**−0.55**	**0.87**	–

MND, myonuclear domain. Bold indicates a significant (P<0.05) correlation after multiple comparison correction using Holm's method.

Table 3. Kruskal–Wallis χ^2 tests examining muscle histology of black-capped chickadees and rock pigeons in relation to heat shock treatment

	Black-capped chickadees			Rock pigeons		
	χ^2	d.f.	P	χ^2	d.f.	P
Fiber diameter	6.54	5	0.26	3.38	5	0.64
Capillary density	0.98	5	0.96	0.84	5	0.97
No. of nuclei	3.92	5	0.56	3.52	5	0.62
MND	1.72	5	0.89	5.75	5	0.33

DISCUSSION

Here, we found that muscle fiber ultrastructure did not change, regardless of the seasonal phenotype, when exposed to heat treatments. All aspects of muscle histology we measured changed significantly across seasons in black-capped chickadees, whereas only capillary density and number of nuclei per fiber changed in rock pigeons. Capillary density did not change as a function of fiber diameter in black-capped chickadees, but capillary density and MND did change with seasonality. Across seasons, as fiber diameter decreased, capillary density increased in the pectoralis muscle of rock pigeons, and capillary density significantly changed with season. For both species in this study, we found that as fiber diameter increased, so did MND.

Phenotypic flexibility is an energy-saving strategy implemented by many wild animals to deal with their changing environment appropriately. It includes reversible traits such as changes in muscle mass (Liknes and Swanson, 2011). Previous studies have demonstrated that muscle mass in both black-capped chickadees and rock pigeon increases relative to body mass in colder months (Vézina et al., 2017; Petit et al., 2014; Saarela and Hohtola, 2003), although others have demonstrated no change in pectoralis muscle mass (Milbergue et al., 2018; Carey et al., 1978). Our data suggest that black-capped chickadees and rock pigeons employ differing phenotypically flexible strategies when dealing with changes across seasons. Black-capped chickadees phenotypically alter their pectoralis muscle structure via hypertrophy in spring relative to summer, which may be an energy-saving strategy due to the higher thermogenic costs associated with a small body mass in colder months (Stager et al., 2015; Milbergue et al., 2018). Larger muscle fiber diameters, as shown in spring phenotype chickadees,

Table 4. Kruskal–Wallis χ^2 tests examining muscle histology of black-capped chickadees and rock pigeons in relation to phenotype (i.e. season when they were captured)

	Black-capped chickadees			Rock pigeons		
	χ^2	d.f.	P	χ^2	d.f.	P
Fiber diameter	7.65	2	0.02	0.12	2	0.94
Capillary density	13.04	2	0.001	45.62	2	<0.001
No. of nuclei	12.71	2	0.002	7.59	2	0.02
MND	13.59	2	0.001	0.53	2	0.77

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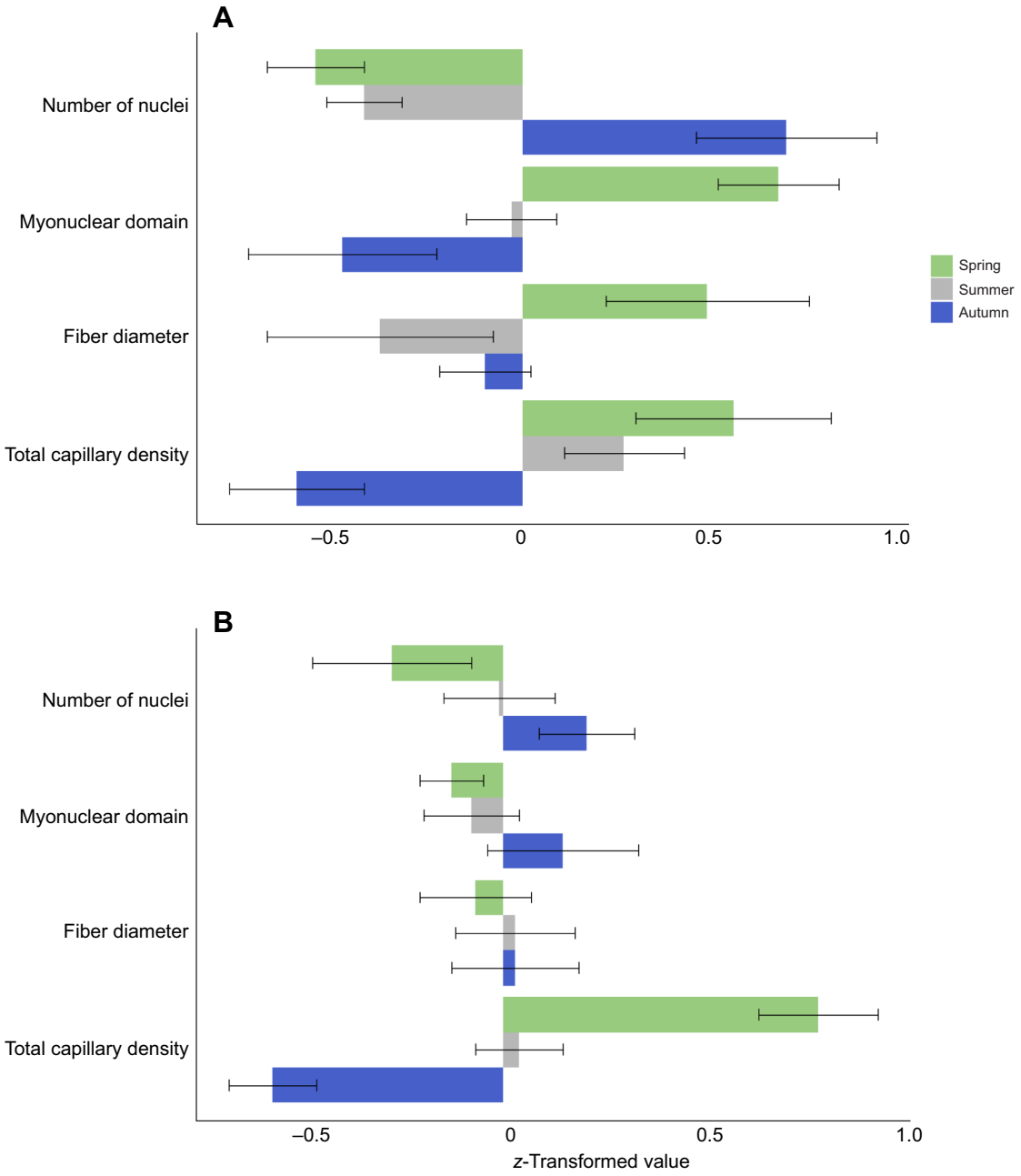

Fig. 2. Muscle histology of the study birds. Individual birds were subjected to either acute or chronic treatment as described in the Materials and Methods and shown schematically in Fig. 1. (A,B) *z*-Transformed mean (±s.e.m.) for black-capped chickadee (A) and rock pigeon (B) muscle histology across phenotypes (spring, summer and autumn). Sample sizes are listed in Table 1. Significant differences are listed in Tables 4–6.

are cheaper to maintain because of the reduced basal metabolic costs associated with the Na^+/K^+-ATPase activity to maintain muscle membrane potential (Jimenez et al., 2013; Jimenez and Williams, 2014). Additionally, increasing muscle fiber diameter during colder months may also explain increases in sustained heat production in winter relative to summer by having muscle fibers that produce more force. Comparatively, rock pigeons also increase their muscle mass at colder acclimation temperatures (Saarela and Hohtola, 2003); however, there is lower selection pressure for rock pigeons to have metabolically cheaper fibers because they do not face the same increased thermogenic capacity or need an increase in force production in colder months as great as that for the small black-capped chickadee. Thus, pigeons may be able to grow their muscle mass via hyperplasia.

Despite changes across seasons, our heat shock treatments for both of the species included in this study demonstrated no

Table 5. *Post hoc* pairwise comparisons of significance from Dunn's test of differences between season and muscle histology in black-capped chickadees

	Fiber	Capillary density	No. of nuclei	MND
Autumn–spring	−1.82	−3.26**	3.36**	−3.68***
Autumn–summer	1.14	−2.78*	2.48*	−1.31
Spring–summer	2.73*	0.39	−0.77	2.16

*P<0.05, **P<0.01, ***P<0.001.

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Table 6. *Post hoc* pairwise comparisons of significance from Dunn's test of differences between season and muscle histology in rock pigeons

	Capillary density	No. of nuclei
Autumn–spring	–6.73***	2.75*
Autumn–summer	–3.28**	1.1
Spring–summer	3.09**	–1.49

*P<0.05, **P<0.01, ***P<0.001.

significant changes across any parameters we measured for any of the seasons we tested. In contrast to our study, winter-phenotype house sparrows thermally challenged for 24 h at 43°C decreased muscle fiber diameter after heat shock, followed by a return to fiber diameters similar to those of the control group in birds that were allowed to recover from the heat shock overnight, pointing to a potential role in temperature regulation of muscle fiber size (Jimenez and Williams, 2014). The lack of response in our birds to increases in temperature to 33°C could, thus, have two implications, neither of which is restrictive or mutually exclusive: (1) 33°C did not induce enough of a thermal challenge to demonstrate the cellular-level changes seen in winter phenotype house sparrows at 43°C, and/or (2) each seasonal phenotype of these birds is thermally plastic enough to cope with large increases in temperature.

Myostatin, a muscle growth inhibitor, seems to regulate muscle growth through hypertrophy and hyperplasia (Lee and McPherron, 2001). Colder temperatures can downregulate the presence of myostatin, which seems to allow the pectoralis muscles of house sparrows to increase in size and mass (Swanson et al., 2009). Therefore, higher levels of myostatin in warm periods could prevent muscle growth and lead to muscle atrophy. However, our results indicate that heat shock to 33°C, either acutely or chronically, did not induce a change in fiber diameter; thus, there may be a maximal thermal set point via which this protein is upregulated. However, *myostatin* gene expression did not change between winter and summer black-capped chickadees (Swanson et al., 2009, 2014; Swanson and Merkord, 2013), whereas others have found that myostatin, a member of module BcCh_M1, was differentially expressed across seasons in chickadees (Cheviron and Swanson, 2017). There was no change in *myostatin* mRNA expression in exercise-trained European starlings (*Sturnus vulgaris*) that increased muscle mass, but there was a significant increase in insulin growth factor-1 (IGF-1) expression (Price et al., 2011). Release of growth hormone from the pituitary increases the cellular production of IGF-1 in tissues (Dantzer and Swanson, 2012). In vertebrates, the insulin receptor regulates energy metabolism, whereas insulin growth factor type I receptor (IGF-1R) promotes growth (Holzenberger et al., 2003). There is also a link between IGF-1 and the activation of hypertrophic muscle growth (Stitt et al., 2004), protein synthesis and satellite cell proliferation (Rennie et al., 2004). Thus, IGF-1 signaling may also be a determinant for muscle growth and temperature sensitive. In white-throated sparrows (*Zonotrichia albicollis*), increases in muscle size could be induced by changes in photoperiod, although in this case, both *myostatin* expression and *IGF1* expression were upregulated simultaneously. The synchrony of these seemingly antagonistic proteins may point to cell size regulation functions (Price et al., 2011) as muscle mass increases. However, more work is needed to understand the ultimate and proximate mechanisms behind muscle fiber diameter change across seasons, including alternative molecular mechanisms (e.g. genes that participate in BMP-signaling; Cheviron and Swanson, 2017).

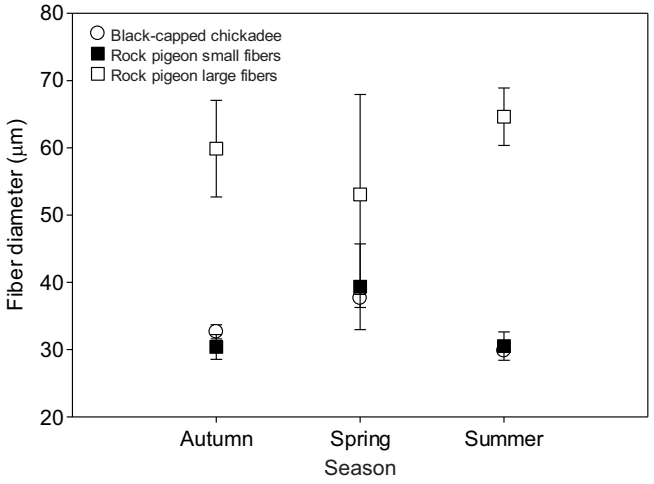

Fig. 3. Changes in mean (±s.e.) fiber diameter across seasons in black-capped chickadees, and both large and small fiber types of rock pigeons. Individual birds were subjected to either acute or chronic treatment as described in the Materials and Methods and shown schematically in Fig. 1. We examined univariate relationships between each variable and treatment as well as each variable and phenotype using Kruskal–Wallis tests. We used Dunn's test for *post hoc* analysis of each significant response variable to examine differences between group levels as implemented in the FSA package (see Materials and Methods) using a corrected α. Black-capped chickadees showed a significant increase in muscle fiber diameter in spring, whereas neither fiber type in the rock pigeons showed changes across seasons. Samples sizes are listed in Table 1. Significant differences are listed in Tables 4–6.

In adult birds, muscle capillarity is independent of body mass (Snyder, 1990) and muscle fibers may be surrounded by a uniform number of capillaries in mammals (Plyley and Groom, 1975), varying by that fiber's metabolic demands. However, it should be noted that others (Bosutti et al., 2015) have determined that capillary density more closely matched fiber size than aerobic potential of the fiber. We found that capillary density was not correlated with fiber diameter in black-capped chickadees, as others have noted (Torrella et al., 1998). This finding is unexpected, as we would assume that as fiber size increases, the demand of oxygen that is supplied by capillaries also increases. Additionally, black-capped chickadees have higher citrate synthase activity in pectoralis muscle in winter relative to summer (Liknes and Swanson, 2011). However, we found that capillary density increased from autumn to spring, potentially demonstrating a mechanism to meet increases in aerobic demand. It may be that angiogenesis, or the process via which endothelial cells extend from an existing capillary to make a new capillary (Haas, 2002), is being signaled through increases in workload due to increases in shivering; however, transcriptomic data on small passerines suggest there is no upregulation of angiogenesis (Cheviron and Swanson, 2017). Muscle overload is often associated with muscle hypertrophy and hyperplasia (Haas, 2002), although vascular responses through muscle stretching or overload flow seem to be minimal (Egginton et al., 1998). Thus, this may imply that more capillaries are not needed to meet the O_2 and ATP demand of cold-acclimated chickadees. Across seasons, as fiber diameter decreased, capillary density increased in the pectoralis muscle of rock pigeons, as previously described (Viscor et al., 1992). In pigeons, cold acclimation increased mitochondrial volume in pectoralis muscle (Mathieu-Costello et al., 1994, 1998). This may imply that increases in oxidative areas are followed by higher capillary density to supply O_2 (Torrella et al., 1996). Angiogenesis can be initiated as a result of low tissue P_{O_2} and has been

demonstrated in hatchling Canada geese (Snyder et al., 1984) and chicken embryos (Strick et al., 1991). Whether this happens solely in early developmental stages remains unclear (Haas, 2002).

Muscle fiber diameter changes have to be compensated for not only with O_2 supply but also with changes in the protein turnover apparatus. Thus, as muscles hypertrophy or atrophy, we assume that there is a change in protein synthesis or degradation, respectively; these changes are monitored by the existing myonuclei in each muscle fiber (Van der Meer et al., 2011). Satellite cells proliferate in existing fibers to maintain this ratio. After post-natal development, satellite cells in birds have been shown to proliferate following stretching (Winchester and Gonyea, 1992). However, in our data, the number of nuclei did not increase in proportion to fiber size, as evidenced by the fact that MND increased significantly with fiber diameter. This finding is consistent with an increase in the MND of white muscle of fishes as a result of an increase in muscle fiber hypertrophic growth (Jimenez and Kinsey, 2012). For both species in this study, we found that as fiber diameter increased, so did MND. This finding seems to suggest that each existing myonucleus in these muscle fibers has to service and maintain protein expression for longer distances. The longer diffusion distances imply that protein transcripts would have to diffuse long distances within each fiber (Van der Meer et al., 2011), and that each myonucleus may be tasked with an increased demand for protein turnover. Increases in MND as a result of muscle hypertrophy can happen to some extent (Van der Meer et al., 2011). It may be that MND is increasing prior to satellite cells being activated and brought into the myofiber. It has been noted that myonuclei are added to a myotube in preparation for a further increase in fiber diameter, and that these myonuclei remain within the fiber even when the fiber atrophies as a preparation for future increases in size (Bruusgaard et al., 2010).

In summary, we found that pectoralis muscle ultrastructure in black-capped chickadees and rock pigeons altered seasonally, possibly as part of their phenotypic plasticity. Black-capped chickadees seem to increase fiber diameter in their spring phenotype, whereas rock pigeons do not alter fiber diameter. This suggests that muscle growth is happening mainly via hypertrophy in black-capped chickadees and hyperplasia in rock pigeons. Heat shock treatments did not impact muscle ultrastructure in either species, suggesting that seasonally acclimatized phenotypes may have a reserve capacity to prevent ultrastructural changes during temperature increases.

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Competing interests
The authors declare no competing or financial interests.

Author contributions
Conceptualization: A.G.J.; Methodology: A.G.J., E.S.O., K.J.B.; Software: A.G.J., E.S.O., K.J.B., C.W.B.; Validation: A.G.J., E.S.O., K.J.B.; Formal analysis: C.W.B.; Investigation: E.S.O.; Data curation: C.W.B.; Writing - original draft: A.G.J., C.W.B.; Writing - review & editing: A.G.J., E.S.O., K.J.B., C.W.B.; Supervision: A.G.J.; Funding acquisition: A.G.J.

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Supplementary information
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Figure S1- Black-capped chickadee fiber diameter, total capillary density and myonuclear domain across seasons and between heat treatments. Data is represented as mean ± SEM.

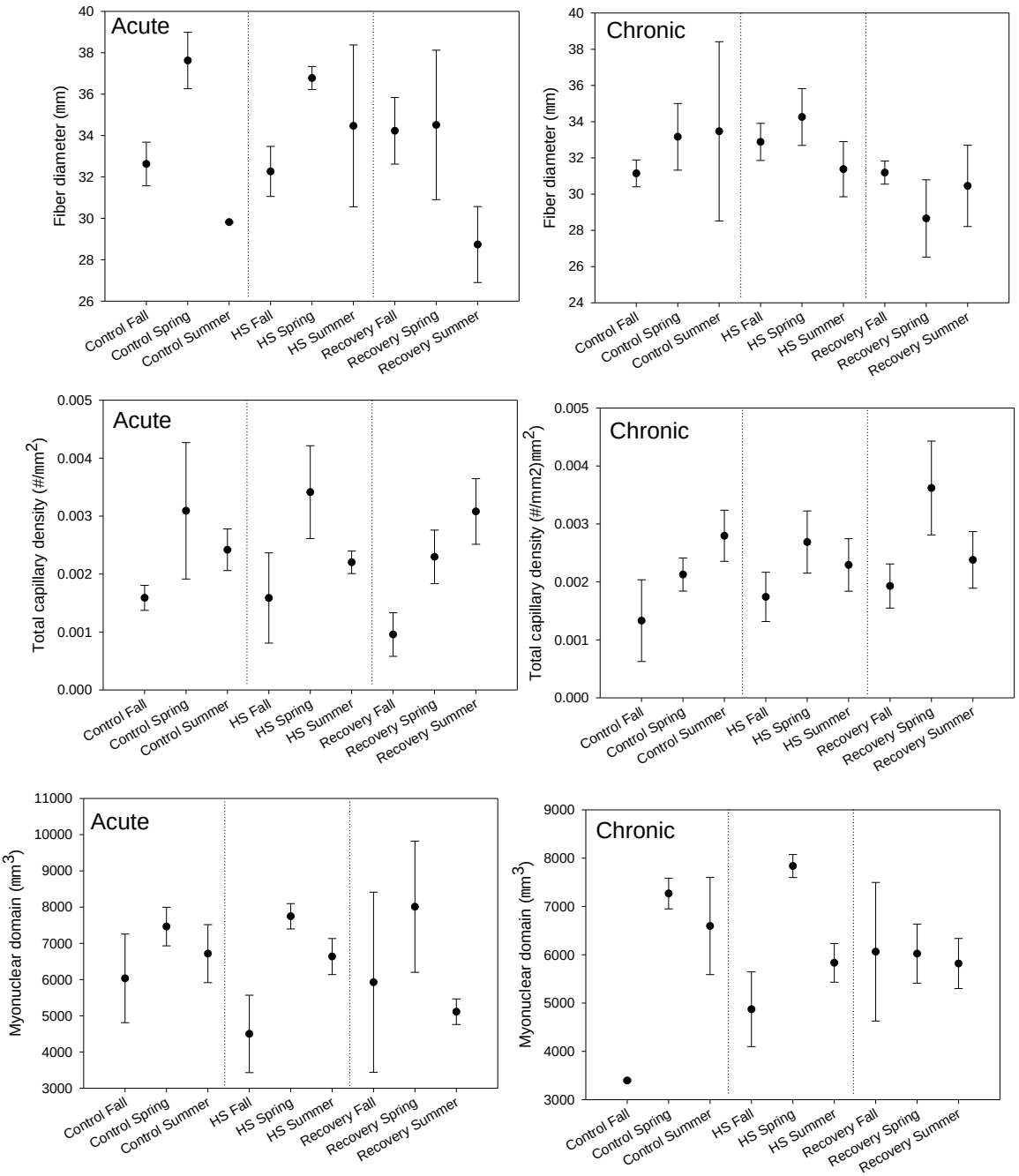

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Figure S2- Rock pigeon fiber diameter, total capillary density and myonuclear domain across seasons and between heat treatments. Data is represented as mean ± SEM.

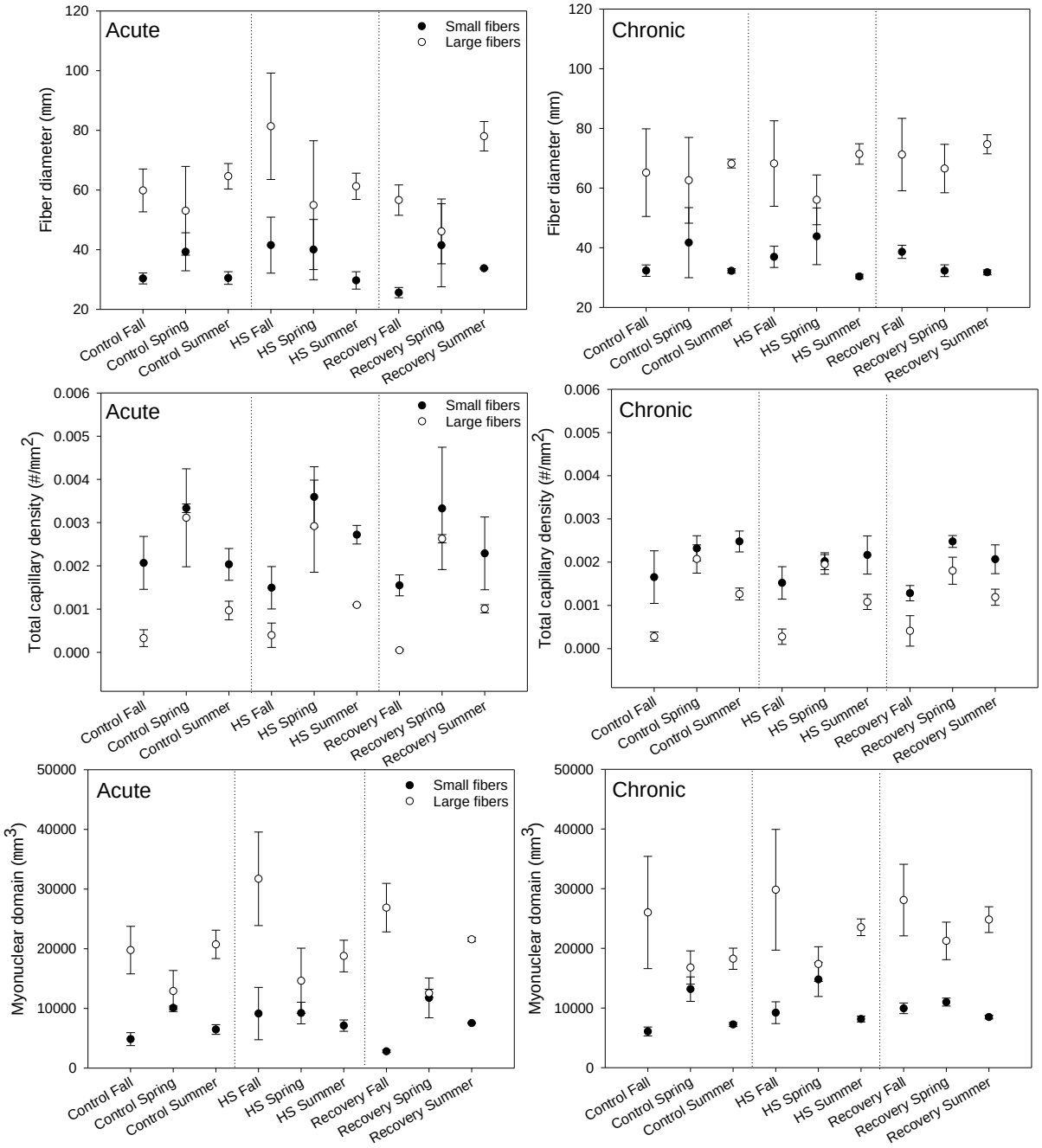

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Figure S3- Scatterplot matrix of black-capped chickadee (above the diagonal) and rock pigeon (below the diagonal) muscle histology across fall, spring and summer. See text for measurement details. Individual birds were collected in Madison County, NY between October 2017 and August 2018. Individuals were sacrificed after 5 days of *ad libitum* food.