Characterizing the influence of chronic hypobaric hypoxia on diaphragmatic myofilament contractile function and phosphorylation in high-altitude deer mice and low-altitude white-footed mice.

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Abstract

Deer mice, *Peromyscus maniculatus,* live at high altitudes where limited O_2 represents a challenge to maintaining oxygen delivery to tissues. Previous work has demonstrated that hypoxia acclimation of deer mice and low altitude white-footed mice (*P. leucopus*) increases the force generating capacity of the diaphragm. The mechanism behind this improved contractile function is not known. Within myocytes, the myofilament plays a critical role in setting the rate and level of force production, and its ability to generate force can change in response to changes in physiological conditions. In the current study, we examined how chronic hypobaric hypoxia exposure of deer mice and white-footed mice influences the Ca^{2+} activation of force generation by skinned diaphragmatic myofilaments, and the phosphorylation of myofilament proteins. Results demonstrate that myofilament force production, and the Ca^{2+} sensitivity of force generation, were not impacted by acclimation to hypobaric hypoxia, and did not differ between preparations from the two species. The cooperativity of the force-pCa relationship, and the maximal rate of force generation were also the same in the preparations from both species, and not impacted by acclimation. Finally, the relative phosphorylation of TnT, and MLC was lower in deer mice than white-footed mice, but was not affected by acclimation. These results indicate that species differences in diaphragm function, and the increase in force production with hypoxia acclimation, are not due to differences, or changes, in myofilament function. However, it appears that diaphragmatic myofilament function in these species is not affected by chronic hypobaric hypoxia exposure.

Introduction

Environmental temperatures and atmospheric oxygen content are lower at high altitudes. For endotherms, these conditions increase metabolic O_2 demands, by increasing thermogenic requirements, while simultaneously restricting tissue O_2 supply (Humphries et al., 2005; McClelland and Scott, 2019). In addition, the metabolic budget of animals is limited by food availability, energy reserves, and how efficiently these fuels can be metabolized (Hammond et al., 1999; Humphries et al., 2005). Therefore, endotherms living at high altitudes must utilize a greater proportion of their energy budget to maintain body temperature, despite the restriction of oxygen availability to support energy production.

The deer mouse (*Peromyscus maniculatus*) has become a useful model for studying the mechanisms of high-altitude adaptation. This species exists over a broad range of altitudes, with populations found below sea level in Death Valley, CA, and up to more than 4300 m above sea level in the Rocky Mountains (Hock, 1964; Natarajan et al., 2015). Many other *Peromyscus* species, such as the closely related white-footed mouse (*P. leucopus*), have morphological, physiological, and ecological similarities to the deer mouse (Feldhamer et al., 1983; Wolff et al., 1985), and may even outcompete deer mice in temperate areas at low altitude (Long, 1996; Wolff, 1985). However, white-footed mice and other *Peromyscus* species are scarce at higher elevations or in colder regions where deer mice are abundant (Bedford and Hoekstra, 2015; Long, 1996). High-altitude populations of the deer mouse can sustain high metabolic rates for prolonged periods in the wild, presumably to support the high cost of thermogenesis in the cold (Conley and Porter, 1986; Hayes, 1989). This suggests that there are morphological and physiological specializations that enable deer mice to thrive in high-altitude environments.

Two defining characteristics of deer mice living at high altitude are their high aerobic capacity for thermogenesis and differences in the control of breathing compared to low altitude conspecifics. High-altitude populations exhibit a higher aerobic capacity (VO_2max) in hypoxia (Cheviron et al., 2012; Cheviron et al., 2013; Lui et al., 2015; Tate et al., 2017) in association with changes in haemoglobin-O_2 affinity, cardiac function, and locomotory muscle phenotype (Lui et al., 2015; Mahalingam et al., 2017; Scott et al., 2015; Snyder, 1985; Storz et al., 2009; Tate et al., 2017; Velotta et al., 2018). The control of breathing at rest has also evolved in high-altitude deer mice, such that highlanders have derived a deep breathing pattern that is unaffected by hypoxia acclimation (Ivy and Scott, 2017, 2018). The diaphragm of high-altitude deer mice also generates less force at a slower rate of development per mg of tissue than the diaphragm from low-altitude white-footed mice (Dawson et al., 2018). This difference in contractile function is not explained by compositional differences, as there was no difference in the relative proportion of the different fibre types (type 1, type IIa, type IIx or type IIb) within the diaphragm between the high altitude and low altitude mice (Dawson et al., 2018). In addition to there being apparent adaptations in diaphragm contractile function in deer mice living at high altitude, the phenotype of the vertebrate diaphragm is also quite responsive to changes in physiological conditions. For example, endurance training causes diaphragm remodelling in laboratory rats, *Rattus norvegicus domesticus,* (Bigard et al., 1992; Goubel and Marini, 1987; Powers et al., 1992), guinea pigs, *Cavia porcellus,* (Lieberman and Maxwell, 1972), humans (Leith and Bradley, 1976; Thayer et al., 2000) and other animals (Gayan-Ramirez and Decramer, 2002). High-altitude hypoxia also increases pulmonary ventilation and thus increases diaphragm activity (Ivy and Scott, 2015; Teppema and Dahan, 2010), which has been demonstrated to induce remodeling of the muscle tissue (Degens et al., 2010; Jammes et al., 1997; Lewis et al., 2016; Shiota et al., 2004). Interestingly, 6-8 weeks of hypobaric

hypoxia acclimation of *Peromyscus* mice, increases the oxidative phosphorylation and force-generation capacities of the diaphragm but did not change the relative proportion, or size, of the different fiber types in the muscle (Dawson et al., 2018). This response is different from that seen in laboratory rats (*Rattus norvegicus domesticus*) where chronic hypobaric hypoxia has been demonstrated to impair diaphragm function as well as affect muscle fibre morphology. For example, hypobaric hypoxia exposure caused a decrease in the force generating capacity, and Ca^{2+} sensitivity of the muscle and affected the endurance capacity of the intact diaphragm (Jammes et al., 1997; McMorrow et al., 2011; Shiota et al., 2004). In addition, Degens et al. (2010) demonstrated that hypobaric hypoxia exposure of laboratory rats caused a decrease in the cross sectional area of type IIa muscle fibers in the diaphragm. A similar decrease in muscle fiber cross-sectional area was found in the diaphragm of the common laboratory mouse, *Mus musculus* with chronic exposure to hypoxia (Gamboa and Andrade, 2012).

One potential explanation for the increase in the contractile capacity of the diaphragm in *Peromyscus* mice that occurs with hypobaric hypoxia acclimation, and for the functional differences between the diaphragms of high-altitude deer mice and low-altitude white-footed mice, is differences in myofilament function (Gehlert et al., 2015; Janssen and Periasamy, 2007). Ca^{2+}-activation of the myofilament initiates the cross-bridge cycle resulting in the generation of force. The functional characteristics of the myofilament, including Ca^{2+} sensitivity, are determined by the complement of contractile proteins that compose it, including troponin (Tn), and tropomyosin (Schiaffino and Reggiani, 2011). Importantly, changes in the Ca^{2+} sensitivity of a myofilament can directly alter the level and rate of force generation by a muscle (For review see (Gordon et al. 2000). Such change can result from phosphorylation of a number of contractile proteins following adrenergic stimulation (Gillis, 2011; Gillis and Klaiman, 2011). For example, phosphorylation of

112 myosin light chain (MLC) modulates the Ca^{2+} sensitivity of skeletal muscle (Frederiksen, 1980;
113 Sweeney and Stull, 1990; Sweeney et al., 1993). Additionally, recent work has demonstrated that
114 reductions in the rate and Ca^{2+} sensitivity of force generation by a multi-fibre diaphragm
115 preparation from a mouse model of heart failure, occurs in parallel with a decrease in the function
116 of the intact diaphragm and in the phosphorylation of desmin, Troponin T (TnT), MLC-1, and
117 MLC-2 (Gillis et al. 2016, Foster et al. 2017). These observations suggest that the differential
118 phosphorylation of the component proteins of the myofilament could contribute to any differences
119 in diaphragmatic contractile function between species or in response to hypoxia acclimation.
120 The purpose of this study was to determine if differences in the Ca^{2+} activation of force
121 generation by diaphragmatic myofilaments could explain the previously observed differences in
122 force generation by the diaphragm muscle between high-altitude deer mice and low-altitude white-
123 footed mice (Dawson et al., 2018), and to uncover any associated changes in the level of
124 phosphorylation of the myofilament proteins. We tested three predictions: (i) myofilaments from
125 highlanders would be less sensitive to Ca^{2+}, have a lower capacity for producing force when
126 maximally activated, and develop force more slowly compared to lowlander myofilaments; (ii)
127 that 6-8 weeks of chronic exposure to hypobaric hypoxia would increase force generating capacity
128 and Ca^{2+} sensitivity of the myofilament, helping explain the previously observed increases in force
129 generation by intact diaphragm muscle after hypoxia acclimation (Dawson et al., 2018); and (iii)
130 the degree of phosphorylation of myofilament proteins will correlate with observed variation in
131 myofilament contractile function.
132
133 **Materials and methods**
134 ***Mouse populations and acclimation treatments***

The populations of mice used in the current study where the same as that in Dawson et al. (2018). In brief, wild adult mice were live-trapped at the summit of Mount Evans at high altitude (Clear Creek County, CO, at 39°35'18''N, 105°38'38'W; 4,350 m above sea level) (*Peromyscus maniculatus rufinus*) and from the Great plains of Nebraska at low altitude (Nine Mile Prairie, Lancaster County, NE, at 40° 52′ 12′′ N, 96° 48′ 20.3′′ W, 430 m above sea level) (*P. leucopus*) and were transported to McMaster University. Mice were then bred within their respective populations to produce first-generation progeny, which were raised to adulthood in standard sea-level (<100 m) holding conditions (barometric pressure of 101 kPa, and O_2 partial pressure of ~20 kPa, 24-25°C, 12:12 light dark photoperiod, food and water *ad libitum*) before experimentation. All animal protocols followed guidelines established by the Canadian Council on Animal Care and were approved by the McMaster University Animal Research Ethics Board.

The mice in this study were the same age range, and were treated identically, as in Dawson et al. (2018). For the treatments, mice from each population (6-12 months of age) were randomly divided into two acclimation groups. The first group continued to be held in standard conditions of normobaric normoxia, while the second group was acclimated to hypobaric hypoxia (barometric pressure of 60 kPa, an O_2 partial pressure of ~12.5 kPa) to mimic the level of hypoxia at ~4,300 m above sea level. Hypobaric hypoxia was maintained using specially designed hypobaric chambers that have been described and used previously (Dawson et al., 2018; Ivy and Scott, 2017a; McClelland et al., 1998). All mice were acclimated for 6-8 weeks. Average ages of the sampled mice for each treatment was 12.0 ± 0.6, 12.8 ± 0.5, 13.1 ± 1.2, and 13.1 ± 1.5 months for normoxic lowlanders, hypoxic lowlanders, normoxic highlanders, and hypoxic highlanders respectively. None of these values were statistically different from each other ($p < 0.05$). Average acclimation

time under hypobaric hypoxic conditions were 6.9 ± 0.3 and 7.0 ± 0.3 weeks for hypoxic lowlander and hypoxic highlander mice respectively.

Muscle mechanics instrument

The force transducer (model 400A, 2.0-kHz resonant frequency, Aurora Scientific), servometer (Model 308C, Aurora Scientific) and fibre test system (Model 802B, Aurora Scientific) was used as described by Gillis et al. (2016). This apparatus from Aurora Scientific (Aurora, ON), was mounted on an inverted microscope (Model Eclipse TE 2000U, Nikon, Japan). The experimental temperature was kept at 15°C ± 1°C using the Aurora Scientific system. A high-speed video sarcomere length system (model 901A, Aurora Scientific) and a CCD camera (model VGA-210-LMCN, Imperx, FL) with 640 x 410 resolution were used to measure sarcomere length.

Diaphragm excision and preparation

Mice were euthanized by isoflurane overdose followed by cervical dislocation, and the diaphragm was subsequently dissected as in Gillis et al. (2016). Diaphragms were then rinsed in a physiological saline (in mM: 94 NaCl, 24 $NaCO_3$, 5 KCl, 1 $MgSO_4$, 1 Na_2HPO_4, and 0.7 $CaCl_2$, pH 7.6 at 15 °C), and then more cleanly dissected on a cooling plate (kept at 4 °C) under a dissection microscope while immersed in a relaxing solution (in mM, 100 KCl, 10 MOPS, 5 dipotassium EGTA, 9 $MgCl_2$, and 4 Na_2ATP, pH 7.0 at 4 °C). The dissected tissues were skinned overnight at 4°C in a skinning solution composed of Triton X-100 (1%), relaxing solution (49.5%), and glycerol (49.5%). This process removes the muscle cell membranes and exposes the myofilament. The skinned muscle was then transferred to a storage solution composed of 50%

relaxing solution and 50% glycerol and was stored at -20 °C. Mechanical measurements were made within 3 days of euthanasia as in Gillis et al. (2016).

Muscle preparations (approximately 2.2 ± 0.05 mm x 0.25 ± 0.007 mm) were then prepared as described in Gillis et al. (2016). Since there can be variation in muscle fibre types throughout the diaphragm, the muscle strips were consistently dissected from the same region of each diaphragm. Preparations were viewed under polarized light to ensure that muscle fibres ran parallel in each preparation. T-clips (Kem-mil, CA) were attached to each end of the strip and were used to mount the preparation onto the force transducer and servo motor via hooks as in Gillis et al. 2016).

Mechanical measurements of diaphragm contraction

The activation solutions used to stimulate muscle contraction were formulated as in Gillis et al. (2015), and composed of the following (in mM): 15 phosphocreatine, 15 EGTA, 40 MOPS, 1 Mg^{2+}, 135 Na^{+}, 135 K^{+}, 1 dithiothreitol, 5 Na_2ATP, approximately 250 units/mL creatine phosphokinase (CPK), and varying concentrations of $CaCl_2$. The Ca^{2+} level (expressed in pCa = -log[molar Ca^{2+}]) varied between pCa 9.0 and 4.8, and were created by adjusting the amount of $CaCl_2$ added as previously described (Gillis et al., 2016). A pre-activation solution containing no $CaCl_2$ (in mM: 100 KCl, 10 MOPS, 0.1 EGTA, 9 $MgCl_2$, and 4 Na_2ATP) was also used.

The Ca^{2+} activation of the preparation was characterized using methods already described (Gillis and Klaiman, 2011; Gillis et al., 2005; Gillis et al., 2016). After the preparation was mounted onto the apparatus, the sarcomere length was measured by analyzing the sarcomere pattern at multiple locations throughout the preparation using FFT as in Gillis and Klaiman, (2011). The sarcomere was set to 2.3 μm by stretching the preparation using the XYZ micrometer translation stage, to

which the servomotor was attached. A sarcomere length of 2.3 μm was used as it is within the range that mouse diaphragm muscle functions (Ribeiro et al., 2013). To standardize force generation between preparations, the cross-sectional area was calculated from the diameter assuming a circular geometry (Gillis and Klaiman, 2011; Gillis et al., 2005; Gillis et al., 2016). At pCa 9, steady state isometric force (fis; passive force) was measured. At all other pCa's, the rate of isometric tension redevelopment (k_{tr}), and Ca^{2+} activated force was measured as described in Gillis et al. (2016).

Sigma-plot (Ver. 12.5) was used to calculate the fis, k_{tr}, and maximum force from the force transducer output. Force-pCa curves were fit to the 4 parameter Hill model:

$$y = y_0 + \frac{ax^b}{c^b + x^b} \quad (1)$$

Where y is the amount of force generated, y_0 is the force generated at rest with no Ca^{2+} present, a is the maximal capacity for force generation, b is the Hill coefficient, c represents the pCa at 50% of maximal force generation (pCa_{50}; halfway between y_0 and a), and x is the pCa. Passive force was subtracted from the force each preparation generated at the respective pCa's. Curves were fit for each preparation and the coefficient of determination was maintained above 0.97. Samples outside of that range were disregarded. 3-4 preparations were measured for each individual, values were then averaged within the individual for statistical tests.

SDS-PAGE gel and phosphorylation detection

Diaphragm samples from each experiment were solubilized using the Precellys Evolution Homogenizer (Bertin Instruments, Montigny-le-Bretonneux) and prepared as in Gillis and Klaiman (2011). 20 μg of each sample was loaded and run at 140 V for ~2.5h on a 12% SDS-PAGE gels using a miniVE system (GE Healthcare). The PeppermintStick Phosphoprotein

(Molecular Probes, Burlington, ON) standard and the Precision Plus Protein Dual Colour (Bio-Rad, California) standard were loaded along with the proteins. Gels were stained with Pro-Q Phosphoprotein Gel Stain (Molecular Probes, Burlington, ON) according to manufacturing instructions. Using a Bio-Rad ChemiDoc MP fluorescent scanner, the gels were imaged. Gels were then stained for total protein content using SYPRO-Ruby Protein Gel Stain (Molecular Probes, Burlington, ON) by the manufacturer's protocol and reimaged with the ChemiDoc.

Protein bands were identified by determining the probable protein size using the standards and matching them to previously determined protein sizes by Gillis et al. (2016). Pro-Q stained gel images were reduced such that only the three phosphorylated protein bands from the PeppermintStick ladder were visible. Bands in the protein lanes that were still visible after this reduction were considered to be phosphorylated. Densitometry was conducted on the images using ImageJ (Fiji; Schindelin et al., 2012). The photo-densities of the phosphorylated bands were quantified and standardized to that of the actin band in the same lane from the SYPRO-ruby stain. This standardization was done to correct for any differences in protein loaded between lanes. Standardized levels of the phosphorylated proteins were then compared between treatment groups.

Statistical methods

Two-way ANOVA followed by post-hoc Holm-Sidak tests was used to test for the main effects of species and acclimation environment and the species×environment interaction on maximum force, passive force, pCa_{50}, Hill's coefficient, and rate of cross-bridge cycling at maximum force generation. Results from protein gel densitometry were standardized to actin content to determine the relative degree of phosphorylation of each myofibrillar protein, which are expressed here relative to the average value of the lowland normoxia group. Two-way ANOVAs

248 and Holm-Sidak tests were similarly performed on the relative degree of phosphorylation.
249 Statistical tests were conducted in SigmaPlot using a significance level of $\alpha = 0.05$.
250

Results

Force production and Ca^{2+} sensitivity

There was no difference in the Ca^{2+}-activated force generated by the diaphragmatic myofilament preparations from the two species (highland deer mice vs lowland white-footed mice) (Figure 1A). High-altitude acclimation resulted in a trend towards greater Ca^{2+}-activated force generation, though not significant (p=0.093). Mean passive force generated by the myofilament preparations did not change with acclimation and was not different between species (Table 1). A regression analysis was performed between maximum force activation and the age of mice and no significant effect of age was found.

Ca^{2+} sensitivity was quantified as the Ca^{2+} concentration required for each preparation to generate 50% maximum force. This concentration, expressed in pCa (pCa=-log[Ca^{2+}]), is called the pCa_{50}. Acclimation environment and species did not impact Ca^{2+} sensitivity (Table 1, Figure 1B). The pCa_{50} was ~5.5 in all groups (Table 1) and there was no effect of acclimation environment or species on force development at pCa 5.5 (Figure 1).

Rate of cross-bridge cycling and cooperativity of Ca^{2+} activated force generation

The rate of cross bridge cycling (k_{tr}) at maximum activation (pCa 5.0) was not different between species, nor acclimation environments (Figure 2, Table 1). k_{tr} increased with pCa, a relationship that was observed in both population and acclimation groups; however, there were no differences in this relationship between the groups (Figure 2). k_{tr} was also plotted against normalized force to examine the relationship between rate of force development and the relative force production (Figure 3). These slopes did not differ between species and acclimation groups

(Table 1). There were no significant main effects of acclimation environment or species on the cooperativity of the force/pCa curve (represented by the Hill coefficient (Table 1).

Protein phosphorylation

Six proteins in the myofilament preparations were found to be phosphorylated: myosin heavy chain (MyHC), myosin binding protein (MyBP), desmin, β-tropomyosin (β-Tm), troponin T (TnT), and myosin light chain (MLC) (Figure 4). The level of phosphorylation did not differ significantly between species or in response to hypoxia acclimation for the myofilament proteins MyHC, MyBP, and β-Tm (Table 2). However, the levels of phosphorylation of TnT and MLC were 41 % and 38 % lower respectively, in highlander diaphragm myofilaments compared to lowlanders (Figure 4), as reflected by a significant ($p < 0.05$) main effect of population but not of acclimation environment in two-way ANOVA (Table 2). The level of phosphorylation of desmin appeared to be lower in highland mice as well, but the main effect of population was not quite significant ($p=0.053$) (Table 2). Acclimation environment had no effects on the degree of phosphorylation for any myofilament proteins. A regression analysis was performed between degree of phosphorylation of each protein and the age of mice and no significant effect of age was found.

Discussion

Maintenance of diaphragmatic myofilament force production and Ca^{2+} sensitivity

The results demonstrate that the Ca^{2+} sensitivity of the multi-fibre diaphragmatic myofilament preparations from the two species were similar, and not affected by acclimation. This suggests that the force generating capacity of the diaphragmatic myofilaments remain relatively invariant. However, the use of a multi-fibre preparation prevents us from characterizing if any differences in the force generating capacity or Ca^{2+} sensitivity of the individual fibre types (Type I, Type IIa, Type IIx, and Type IIb) found in the muscle varied between species or were affected by the different treatments. Previous studies with lab rats, have demonstrated that the Ca^{2+} sensitivity of force generation varies between fiber types, and that this can differentially change within fiber types during development, or in response to experimental manipulation (Geiger et al. 1999; Geiger et al. 2000; Geiger 2001a; Geiger 2001b). Such a response would alter the contractile function of the diaphragm. However, as mentioned earlier, we have demonstrated, using a multi-fibre myofilament preparation, that a decrease in diaphragm function, observed in a mouse model of heart failure, is reflected in a decrease in Ca^{2+} sensitivity and force generating capacity of the preparation (Gillis et al. 2016). Together, these factors suggest that the results of the current study provide insight into the summed functional characteristics of the diaphragmatic myofilament but that the use of the multi-fiber preparation may have obscured any differences, or changes in the function of individual fiber types.

The slight, but not significant, increase in maximum force generation with hypoxia acclimation suggests that the myofilament could play a role in the increase in force production by the intact diaphragm with hypoxia acclimation (Dawson et al. 2018) but that differences in Ca^{2+} handling by the sarcoplasmic reticulum are primarily responsible. Furthermore, the absence of

similar effects of chronic hypobaric hypoxia in our current and previous studies suggest that the contractile function and morphology of the diaphragm myofilaments in *Peromyscus* mice are more resistant to chronic hypoxia than those of laboratory mice and rats, discussed earlier. Considering that impairments in the force generating capacity of the diaphragm in chronic hypoxia are somewhat similar to the changes that can occur in patients with COPD (Chronic Obstructive Pulmonary Disease) (Lewis and O'Halloran, 2016; Polla et al., 2004), the symptoms of which have been linked with chronic intermittent hypoxaemia (Raguso et al., 2004), suggest that *Peromyscus* mice may be a useful model for examining how these impairments are avoided.

Rate of cross-bridge cycling and cooperativity of force-[Ca^{2+}] exhibited differences between species

The rates of cross-bridge cycling in maximally activated diaphragm myofilaments did not differ as a result of species or acclimation. Thus, the differing rates of force development that have been previously observed using intact diaphragm muscle from acclimation and species (Dawson et al., 2018) cannot be attributed to changes in the myofilament. Previous work has similarly demonstrated that acclimation of laboratory rats to hypobaric hypoxia has no effect on cross-bridge cycling in diaphragm myofilaments (Degens et al., 2010; El-Khoury et al., 2003; Shiota et al., 2004). The results of this study suggest that it may be changes to Ca^{2+}-handling within the intact muscle that is responsible for the effects of acclimation on force development.

The cooperativity of Ca^{2+} activation of force development did not change with hypoxia acclimation in either species, resulting in similar levels observed in the preparations from lowland mice, regardless of acclimation. This suggests that the influence of the activation of a single functional unit within the myofilament (7 actin, 1 troponin complex, 1 tropomyosin; Gillis et al.,

2007) on the activation of adjacent functional units within the same myofilament, is not affected by hypoxia nor is it different between species.

Myofilament protein phosphorylation

In cardiac muscle, phosphorylation of troponin I (TnI) and TnT is a critical mechanism by which myofilament activation, and therefore muscle contractile function, is regulated (Colson et al., 2008; Dong et al., 2007; Layland et al., 2005; Messer et al., 2007; Noland and Kuo, 1993; Salhi et al., 2014). Skeletal muscle contractile function can also be manipulated through phosphorylation of some of the same proteins, such as MLC, TnT, and desmin, but to a lesser degree (Sweeney and Stull, 1990; Sweeney et al., 1993). For example, phosphorylation of MLC has been shown to increase the Ca^{2+} sensitivity, the rate of force development and the maximum force generated by skeletal muscle (Hodgson et al., 2005; Sweeney and Stull, 1990; Sweeney et al., 1993). Similarly, we have previously reported that decreases in phosphorylation of MLC 1 and MLC 2, as well as TnT and desmin, correlate with decreases in the Ca^{2+} sensitivity, maximal force generation, and rate of force redevelopment in a multi-fibre, diaphragmatic myofilament preparation in a study of a mouse model of heart failure (Gillis et al., 2016). Here, we show that lower levels of TnT and MLC phosphorylation in diaphragm myofilaments of highlanders compared to lowlanders were not associated with any significant changes in functional parameters between species. These results suggest that phosphorylation state has no effect on Ca^{2+} sensitivity, maximal force generation, or rate of force generation, at least in *Peromyscus* mice, or alternatively that effects on these aspects of myofilament function were compensated for by other differences in the myofilament proteins.

Perspectives

One question remaining is, could there be a benefit of high-altitude deer mice having a diaphragm with a comparatively low force generating capacity (Dawson et al., 2018)? Since the diaphragm rarely uses its full contractile potential, and fast-contracting fibre types are only recruited when large amounts of force are required (e.g., sneezing and coughing) (Fogarty et al., 2018; Mantilla et al., 2010; Sieck and Fournier, 1989), a reduction in the diaphragm's capacity for force generation is unlikely to constrain pulmonary ventilation. The reduction in diaphragm contractility could instead act to enhance thermogenesis to cope with the cold environment at high altitudes. Although our current results suggest that highlanders have retained a normal capacity for force generation by the myofilament, highlanders have higher activities of sarcoplasmic reticulum (SR) Ca^{2+}-ATPase (SERCA) in the diaphragm. Higher SERCA activity could increase the rate of reuptake of Ca^{2+} into the SR and limit the rise in intracellular Ca^{2+} ($[Ca^{2+}]_i$) during excitation. Given that mammalian skeletal muscles normally operate at $[Ca^{2+}]_i$ below that at pCa_{50} (Gehlert et al., 2015), then modest changes in $[Ca^{2+}]_i$ arising from alterations in SERCA activity could have a limiting effect on force production during contraction, thereby explaining the differences in force production by the intact muscle (Dawson et al., 2018). Additionally, increasing active Ca^{2+} reuptake could also augment Ca^{2+} cycling, ATP hydrolysis, and thus heat production by the diaphragm. This mechanism of thermogenesis would be similar (albeit arising from a far less extreme phenotype) to the futile Ca^{2+} cycling seen in the extraocular heater organ of marlins and other billfish that generates heat by high levels of ATP hydrolysis (Block, 1994; Block et al., 1994). The location of the diaphragm, next to many vital organs, could make it especially effective as a source of supplemental heat production.

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602 ***TABLES***

603 **Table 1.** Measured variables resulting from the Ca^{2+} activation of myofilament preparations from the diaphragms of highland deer mice
604 (*Peromyscus maniculatus*) and lowland white-footed mice (*P. leucopus*). Data are means ± SEM. F_{max}, Ca^{2+} activated maximum force
605 generated by diaphragm myofilaments. pCa_{50}, Ca^{2+} concentration, reported as pCa, required for the diaphragm myofilament to reach
606 50% maximum force. n_H, Hill coefficient. Passive Force, force generated at pCa 9.0. k_{tr} at F_{max}, Rate of cross bridge cycling at maximum
607 activation. slope of the k_{tr}-force relationship, slope of line fit to the relationship between k_{tr} and normalized force generation. Data
608 calculated by averaging 3-4 preparations per individual. Number of individuals as follows: normoxic lowlanders, 7; hypoxic lowlanders,
609 7; normoxic highlanders, 7; hypoxic highlanders, 8. There was no significant differences between values in the same column as tested
610 using a two-way ANOVA ($p < 0.05$).

Group	F_{max} (mN/mm^2)	pCa_{50}	n_H	Passive Force (mN/mm^2)	k_{tr} at F_{max} (s^{-1})	k_{tr} - force slope
Normoxic Lowlanders	30.322 ± 2.238[a]	5.518 ± 0.0109[a]	7.160 ± 0.627[a]	1.262± 0.0540[a]	6.005 ± 0.228[a]	5.040 ± 0.353[a]
Hypoxic Lowlanders	36.960 ± 3.981[a]	5.524 ± 0.00692[a]	7.572 ± 0.556[a]	1.218 ± 0.165[a]	6.221 ± 0.583[a]	5.478 ± 0.620[a]
Normoxic Highlanders	32.142 ± 3.142[a]	5.523 ± 0.00375[a]	6.661 ± 0.576[a]	1.502 ± 0.189[a]	5.383 ± 0.232[a]	4.317 ± 0.218[a]
Hypoxic Highlanders	37.760 ± 4.298[a]	5.517 ± 0.0108[a]	7.819 ± 0.515[a]	1.486 ± 0.313[a]	5.802 ± 0.295[a]	5.178 ± 0.280[a]

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612

613 **Table 2.** Two-way ANOVA results of contractile function of myofilaments and of relative phosphorylation of myofilament proteins.

	Population main effect	Environmental main effect	Interaction
Functional data (data in Fig. 1-3)			
Maximum force produced	$F_{1,82}$= 3.036, p= 0.093	$F_{1,82}$= 0.0178, p= 0.895	$F_{1,82}$= 0.0963, p= 0.759
Passive force produced (fis)	$F_{1,82}$= 0.0198, p= 0.889	$F_{1,82}$= 1.447, p = 0.240	$F_{1,82}$ = 0.00461, p = 0.946
pCa_{50}	$F_{1,82}$= 0.0315, p= 0.860	$F_{1,82}$= 0.0601, p= 0.808	$F_{1,82}$ = 0.687, p = 0.415
Force at pCa_{50}	$F_{1,82}$= 2.025, p= 0.167	$F_{1,82}$= 0.0388, p= 0.845	$F_{1,82}$ = 0.380, p = 0.543
k_{tr} at F_{max}	$F_{1,82}$ = 0.692, p = 0.413	$F_{1,82}$ = 1.852, p = 0.185	$F_{1,82}$ = 0.0707, p = 0.792
Hill's coefficient	$F_{1,82}$ =1.912, p = 0.179	$F_{1,82}$ =0.0494, p = 0.826	$F_{1,82}$ = 0.431, p = 0.517
k_{tr}-normalized force slope	$F_{1,82}$ = 3.115, p = 0.089	$F_{1,82}$ = 1.128, p = 0.298	$F_{1,82}$ = 0.708, p = 0.408
Phosphorylation data (data in Fig. 4)			
MyHC	$F_{1,23}$ = 1.224, p = 0.283	*$F_{1,23}$ = 1.058, p = 0.317*	*$F_{1,23}$ = 0.319, p = 0.579*
Desmin	*$F_{1,23}$ = 4.167, p = 0.053*	*$F_{1,23}$ = 0.274, p = 0.605*	*$F_{1,23}$ = 0.0357, p = 0.852*
β-Tm	*$F_{1,23}$ = 0.125, p = 0.726*	*$F_{1,23}$ = 0.217, p = 0.645*	*$F_{1,23}$ = 0.173, p = 0.681*
TnT	*$F_{1,23}$ = 4.763, p = 0.041*	*$F_{1,23}$ = 0.914, p* = 0.350	*$F_{1,23}$ = 1.383, p = 0.253*
Holm-sidak post-hoc:	Within highland: p = 0.880	Within hypoxia: p = 0.476	
	Within lowland: p = 0.139	Within normoxia: p = 0.030	
MLC	*$F_{1,23}$ = 6.004, p* = 0.022	*$F_{1,23}$ = 0.613, p = 0.441*	*$F_{1,23}$ = 0.345, p = 0.562*
Holm-sidak post-hoc:	Within highland: p = 0.893	Within hypoxia: p = 0.207	
	Within lowland: p = 0.334	Within normoxia: p = 0.039	

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FIGURE LEGENDS

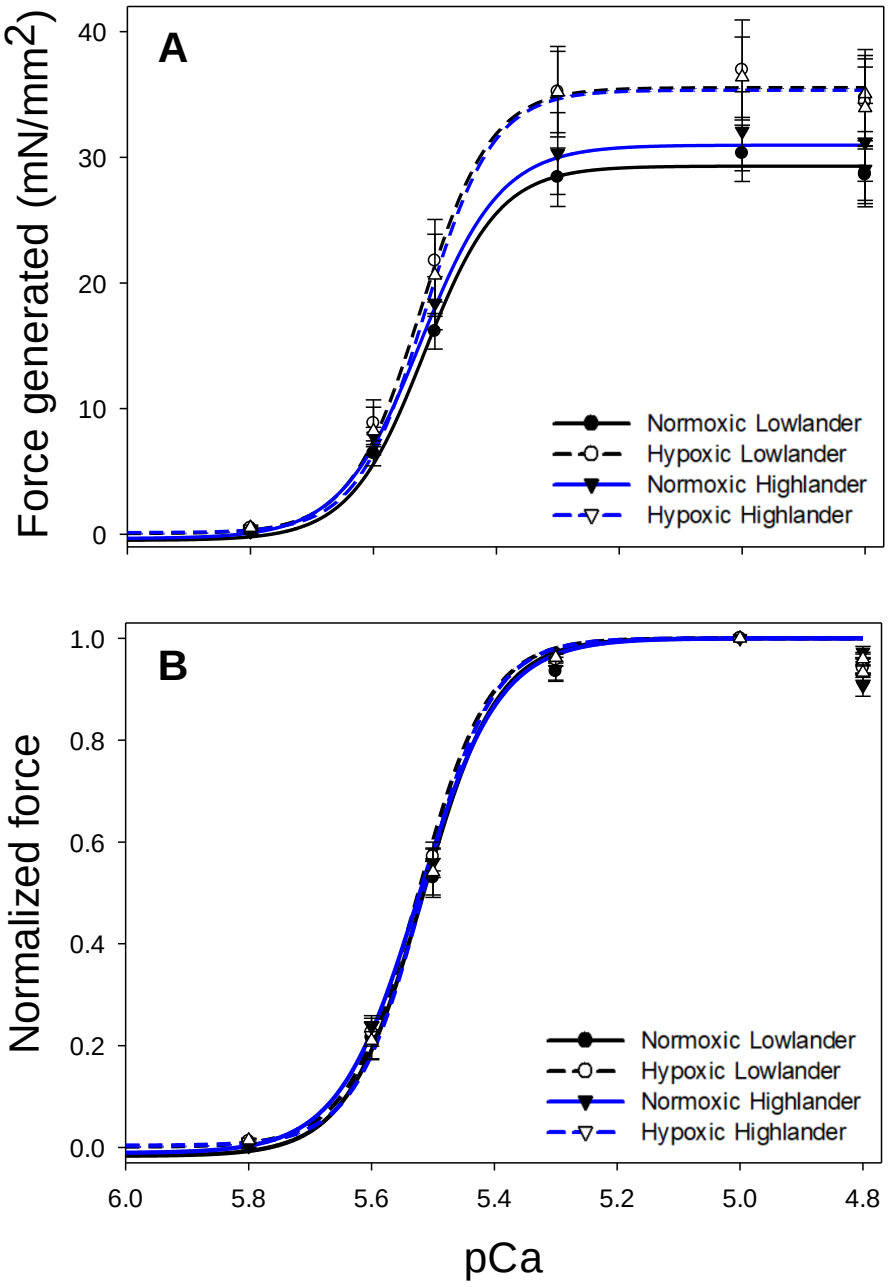
Figure 1. Hypobaric hypoxia had no effects on maximum force generation or Ca^{2+} sensitivity of diaphragmatic myofilaments from highland deer mice (*Peromyscus maniculatus*) and lowland white-footed mice (*P. leucopus*). Absolute Ca^{2+}-activated force generation (A) and normalized force generation (B) of myofilament preparations. Normalized forces were calculated relative to the average maximum force capacity produced by each group (upper force plateau in panel A). Data are means ± SEM. Regression curves were generated by fitting data to a 4 parameter Hill equation. Treatment means were calculated by averaging data from 3-4 preparations per individual and then taking the mean of these values. Number of individuals as follows: normoxic lowlanders, 7; hypoxic lowlanders, 7; normoxic highlanders, 7; hypoxic highlanders, 8. No significant differences were found between groups within a pCa.

Figure 2. The influence of Ca^{2+} concentration on cross-bridge cycling, measured as k_{tr}, and steady state isometric force generation in diaphragmatic myofilaments from highland deer mice (*Peromyscus maniculatus*) and lowland white-footed mice (*P. leucopus*) acclimated to hypobaric hypoxia. Plots A and B show effects of acclimation environment for lowland mice (A) and highland mice (B). Plots C and D compare between species under normoxic conditions (C) and hypoxic conditions (D). Number of mice in each group are shown in Figure 1.

Figure 3. Influence of hypobaric hypoxia on the relationship between rate of cross-bridge cycling and normalized force generation of diaphragmatic myofilaments of highland deer mice (*Peromyscus maniculatus*) and lowland white-footed mice (*P. leucopus*). Plots A and B show effects of acclimation environment for lowland mice (A) and highland mice (B). Plots C and D compare between species under normoxic conditions (C) and hypoxic conditions (D). (E) Mean ±

SEM of the slopes generated from linear regressions between rate of cross bridge cycling and normalized force generation in A-D. Number of mice in each group are shown in Figure 1.

Figure 4. Influence of hypobaric hypoxia on the phosphorylation of diaphragmatic myofilaments from highland deer mice (*Peromyscus maniculatus*) and lowland white-footed mice (*P. leucopus*). (A) SYPRO-Ruby indicating total protein and (B) Pro-Q diamond stained gels indicating phosphorylated protein along with sizes indicated by the PeppermintStick protein standard. Images contain lanes from 3 different gels. (C) Relative level of phosphorylation (mean ± SEM) of myofilament proteins. The levels of phosphorylation in each group were standardized to the average level of phosphorylation in the lowland normoxic group. Bands were identified as previously described (Gillis et al., 2016). Normoxic lowlander (n=8), hypoxic lowlander (n=7), normoxic highlander (n=8), hypoxic highlander (n=8). Where n is the number of individual diaphragms sampled. * represents significant main effects of species in two-way ANOVA ($p < 0.05$)



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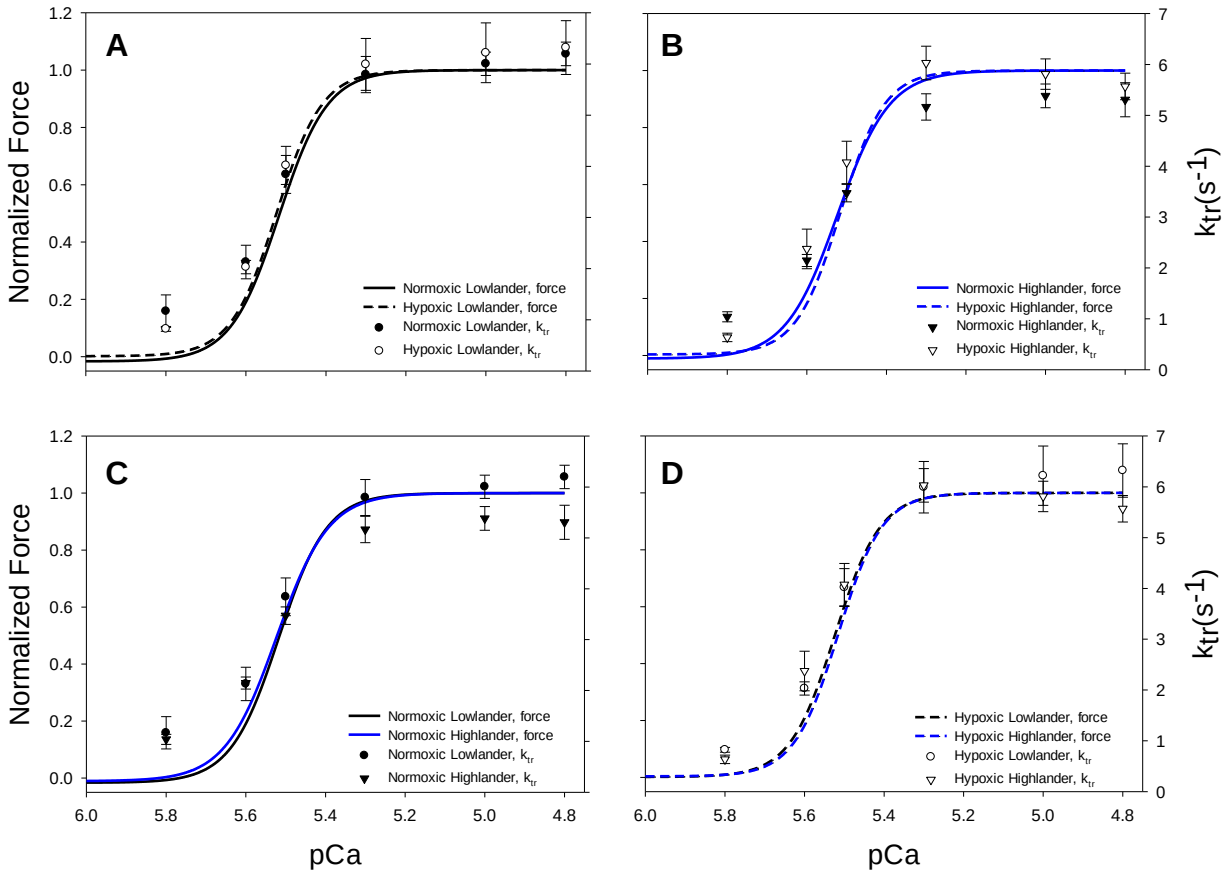

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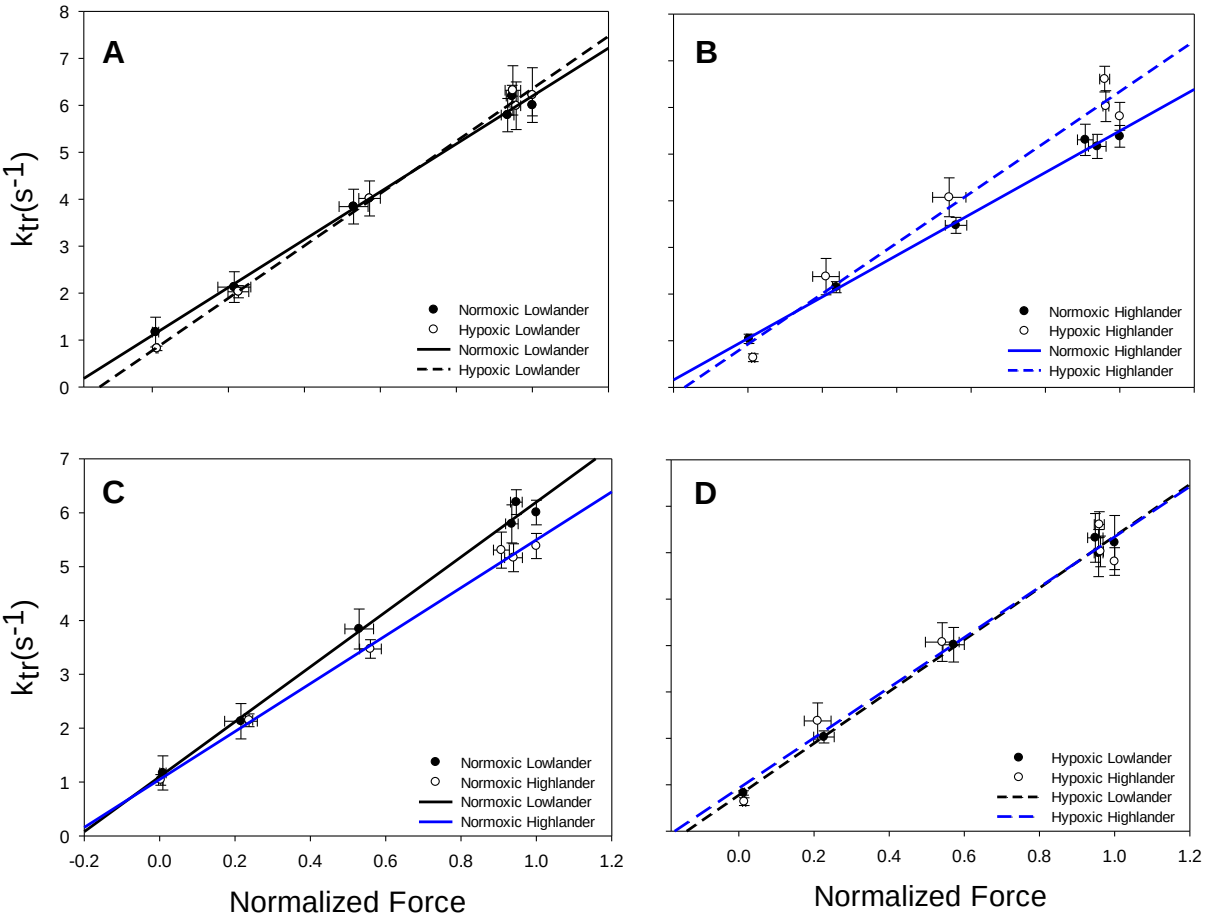

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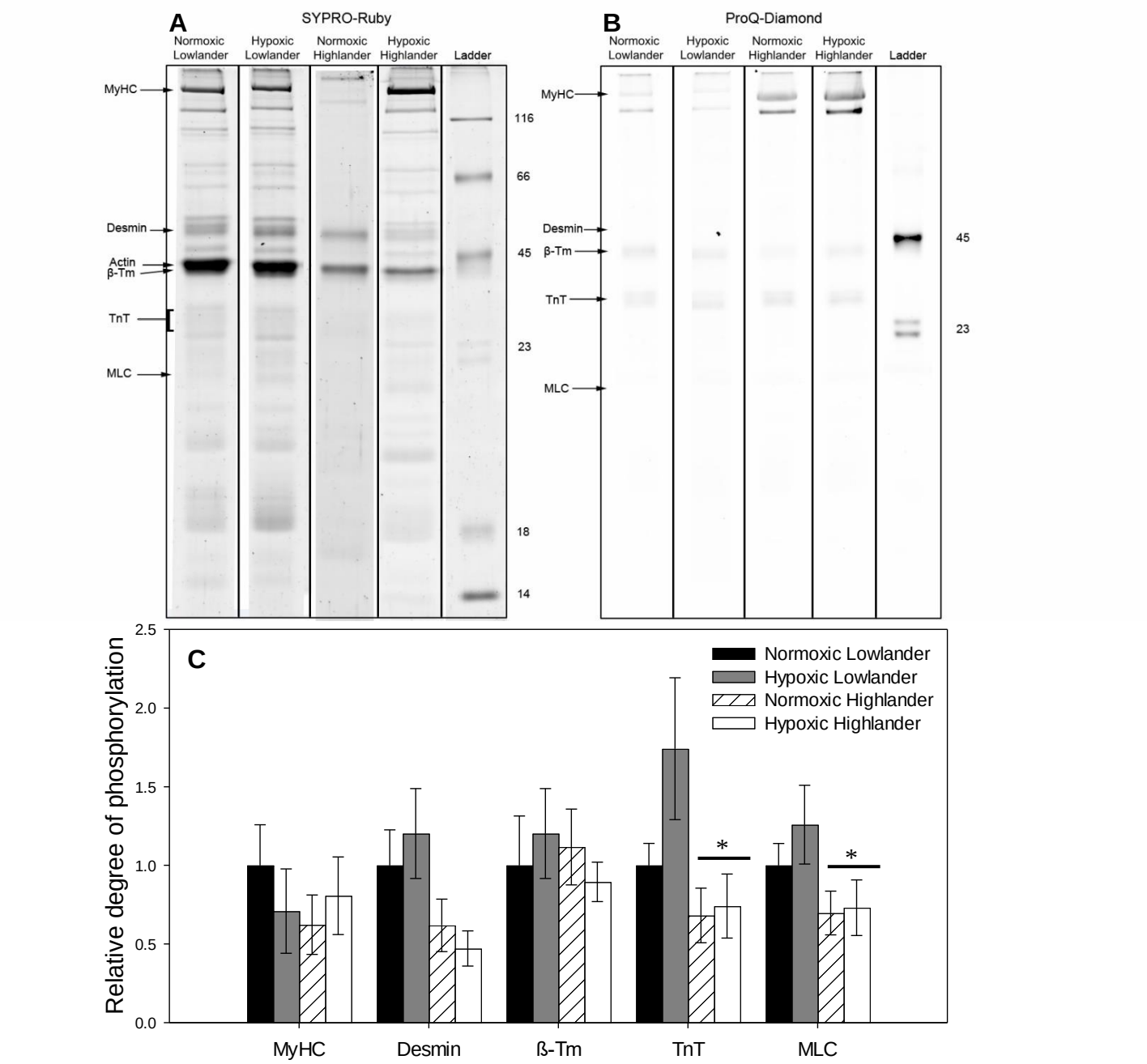

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