The Mitochondrial Basis for Adaptive Variation in Aerobic Performance in High-Altitude Deer Mice

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Synopsis

Mitochondria play a central role in aerobic performance. Studies aimed at elucidating how evolved variation in mitochondrial physiology contributes to adaptive variation in aerobic performance can therefore provide a unique and powerful lens to understanding the evolution of complex physiological traits. Here, we review our ongoing work on the importance of changes in mitochondrial quantity and quality to adaptive variation in aerobic performance in high-altitude deer mice. Whole-organism aerobic capacity in hypoxia (VO_2max) increases in response to hypoxia acclimation in this species, but high-altitude populations have evolved consistently greater VO_2max than populations from low altitude. The evolved increase in VO_2max in highlanders is associated with an evolved increase in the respiratory capacity of the gastrocnemius muscle. This appears to result from highlanders having more mitochondria in this tissue, attributed to a higher proportional abundance of oxidative fibre-types and a greater mitochondrial volume density within oxidative fibres. The latter is primarily caused by an over-abundance of subsarcolemmal mitochondria in high-altitude mice, which is likely advantageous for mitochondrial O_2 supply because more mitochondria are situated adjacent to the cell membrane and close to capillaries. Evolved changes in gastrocnemius phenotype appear to be underpinned by population differences in the expression of genes involved in energy metabolism, muscle development, and vascular development. Hypoxia acclimation has relatively little effect on respiratory capacity of the gastrocnemius, but it increases respiratory capacity of the diaphragm. However, the mechanisms responsible for this increase differ between populations: lowlanders appear to adjust mitochondrial quantity and quality (i.e., increases in citrate synthase [CS] activity, and mitochondrial respiration relative to CS activity) and they exhibit higher rates of mitochondrial release of reactive oxygen species (ROS), whereas

highlanders only increase mitochondrial quantity in response to hypoxia acclimation. In contrast to the variation in skeletal muscles, the respiratory capacity of cardiac muscle does not appear to be affected by hypoxia acclimation and varies little between populations. Therefore, evolved changes in mitochondrial quantity and quality make important tissue-specific contributions to adaptive variation in aerobic performance in high-altitude deer mice.

Introduction

Elucidating the mechanistic basis of adaptive variation in organismal performance is a key goal of evolutionary physiology (Garland and Carter 1994; Dalziel et al. 2009). Aerobic performance, such as that exhibited in endotherms during intense exercise or cold-induced thermogenesis (heat generation), is a complex trait that involves the coordinated function of several physiological systems. Mitochondria play a central role in aerobic performance, as the ultimate consumer of O_2 and metabolic fuels during the process of aerobic energy production *via* oxidative phosphorylation (OXPHOS). Mitochondria in active muscles are commonly believed to consume O_2 at near maximal rates *in vivo* when animals exercise at their whole-organism aerobic capacity (maximal O_2 consumption rate, VO_2max) (Schwerzmann et al. 1989; Suarez et al. 1991). Understanding the mitochondrial basis for adaptive variation in aerobic performance can therefore provide a unique and powerful lens into the underlying mechanisms for the evolution of complex physiological traits.

It has long been appreciated that variation in aerobic performance is associated with variation in the respiratory and/or mitochondrial phenotypes of active tissues (e.g., muscles involved in locomotion or shivering). Mitochondrial quantity – or more specifically, mitochondrial volume density – can vary appreciably across cell types (e.g., between oxidative and glycolytic muscle fibre types), and can also vary in similar cell types between species (Mathieu et al. 1981; Scott et al. 2009a). Variation in the mitochondrial quantity of active tissues has often been associated with variation in VO_2max (Schwerzmann et al. 1989; Weibel et al. 2004; Weibel and Hoppeler 2005). However, the manner in which mitochondria work is also a key determinant of tissue respiratory capacity, and there is a growing appreciation that

mitochondrial quality can change in a variety of situations (e.g., changes in respiratory control and/or capacity of a given volume of mitochondria) (Jacobs and Lundby 2013; Hepple 2016). There is ongoing debate about whether changes in mitochondrial quality in active tissues contribute to variation in aerobic performance (Gnaiger 2009; Jacobs and Lundby 2013; Hepple 2016). Nevertheless, relatively few studies have elucidated the combined importance of mitochondrial quantity and quality in adaptive evolutionary variation in aerobic performance between populations or species.

North American deer mice (*Peromyscus maniculatus*) are an excellent model species for examining the mitochondrial basis for adaptive variation in aerobic performance. Their native altitudinal range extends from around sea level to over 4300 m elevation in the Rocky Mountains (Snyder et al. 1982; Natarajan et al. 2015), and high-altitude populations must sustain high metabolic rates in the wild to support thermogenesis in cold alpine environments (Hayes 1989). Environmentally-induced plasticity, such as occurs during hypoxia acclimation in the lab or natural acclimatization to high-altitude environments, augments VO_2max in this species (Chappell et al. 2007; Cheviron et al. 2012; Cheviron et al. 2013; Lui et al. 2015; Tate et al. 2017). High-altitude populations have also evolved a higher VO_2max in hypoxia than low-altitude populations (Cheviron et al. 2012; Cheviron et al. 2013; Lui et al. 2015; Tate et al. 2017), likely as an adaptation to strong directional selection on VO_2max in the wild (Hayes and O'Connor 1999). Our recent work suggests that variation in mitochondrial physiology contributes to this adaptive variation in aerobic performance in high-altitude deer mice. Here, we will start with a discussion of the various approaches that can be used to study this issue. We will then review our work on high-altitude deer mice to examine the relative importance of mitochondrial quantity and quality to adaptive variation in aerobic performance.

Measuring mitochondrial content and respiratory capacity

Several techniques can provide insight into the quantity of mitochondria in tissues. Conventional transmission electron microscopy has been a powerful tool for measuring mitochondrial volume density and morphology for several decades, and can now be extended with electron tomography to reconstruct three-dimensional mitochondrial structure (Mathieu et al. 1981; Marín-García 2013). Recent developments in fluorescence and super-resolution microscopy techniques and analysis are advancing the potential for imaging mitochondrial structure and dynamics in live cells (Picard et al. 2011; Lidke and Lidke 2012; Jakobs and Wurm 2014). In addition to these imaging techniques, indirect markers of mitochondrial volume such as citrate synthase (CS) activity and others (activity of mitochondrial complexes I-IV, sarcolipin content, etc.) can provide useful insight into the mitochondrial content of cells and tissues (Reichmann et al. 1985; Larsen et al. 2012). These approaches are useful for examining the potential for evolved and/or environmentally-induced variation in mitochondrial quantity in tissues.

Mitochondrial quality is typically examined by measuring the respiratory function of tissues and mitochondria, which can be accomplished using several types of mitochondrial preparations. Mitochondria can be isolated from other components of the cell by mechanical homogenization and differential centrifugation (Frezza et al. 2007). These isolated mitochondria preparations have been used for decades and have led to several foundational discoveries about mitochondrial function (Chance and Williams 1955; Mitchell 1961; Williams 1965), and they continue to be valuable when precise experimental control is needed and/or when diffusion

limitation and interference from cytosolic factors must be minimized (Brand and Nicholls 2011). However, it has more recently become clear that the function of isolated mitochondria may not always reflect the function of mitochondria *in situ*, because isolation methods may select for a biased sub-population of mitochondria (e.g., the healthiest or least fragile), they can disrupt the often complex architecture of mitochondria in intact cells, or they may otherwise alter mitochondrial function (Saks et al. 1998; Picard et al. 2010; Picard et al. 2011). Mitochondrial preparations obtained from cells or tissues by mechanically and/or chemically permeabilizing the cell membrane do not suffer from these limitations, because they preserve the functional and structural integrity of the mitochondria (e.g., permeabilized fibres or cells, tissue homogenates) (Kuznetsov et al. 2008; Larsen et al. 2014). Ultimately, the choice between these mitochondrial preparations is a balance between experimental control and physiological relevance.

High-resolution respirometry can be used to examine respiratory capacities for OXPHOS and mitochondrial electron transport in the mitochondrial preparations discussed above. The respiration of mitochondria is often measured as the consumption of O_2 in a small closed chamber, often in a medium with optimal pH and ionic conditions and with other substances that support good mitochondrial function (e.g., membrane stabilizers, antioxidants, etc.). Mitochondrial respiration can be measured after sequential substrate, uncoupler, and inhibitor titrations ('SUIT' protocol) to stimulate OXPHOS and/or electron transport with electron entry *via* single or multiple complexes in the electron transport system (ETS) (Pesta and Gnaiger 2012). An example of such a protocol is shown in Figure 1, using mitochondria isolated from gastrocnemius muscle (A) or heart ventricle (B) of an individual deer mouse. Measuring respiration in well-coupled mitochondria (solid lines in Fig. 1) can be used to determine the maximum physiological capacity for OXPHOS (i.e., respiration supported by convergent

electron input *via* complexes I and II), and for determining the relative OXPHOS capacities of each mitochondrial complex. Alternative substrates can also be chosen (e.g., pyruvate versus fatty acyl carnitines) to evaluate differences in the capacity to oxidize particular metabolic fuels.

The phosphorylation system (i.e., ATP synthase, adenine nucleotide translocase, and inorganic phosphate transporter) can have some restraining control over OXPHOS, such that respiration measurements in well-coupled mitochondria may not represent the full capacity of the ETS (Gnaiger 2009; Pesta and Gnaiger 2012). Mitochondrial respiratory control by the phosphorylation system can be released by experimentally titrating an exogenous protonophore (dashed lines in Fig. 1), and in this non-coupled state, measurements of mitochondrial respiration reflect the full capacity of the ETS. The relative influence of the phosphorylation system, and how it can differ between mitochondria from different tissues, is illustrated in Figure 1 for an individual deer mouse. In mitochondria from the gastrocnemius (Fig. 1A), ETS capacity is extremely similar to OXPHOS capacity across a range of substrate combinations, but in those from the heart ventricle (Fig. 1B), ETS capacity is higher than OXPHOS capacity. This suggests that the phosphorylation system has a restraining influence on mitochondrial respiration in the heart, but not in the gastrocnemius. Control by the phosphorylation system may also vary between species or in different conditions, and may even be adjusted to help offset deficiencies in the ETS (Gnaiger 2009; Porter et al. 2015; Du et al. 2017), so in some situations it may be necessary to determine the magnitude of control by the phosphorylation system in order to fully appreciate the factors affecting mitochondrial respiration.

SUIT protocols can be used with different mitochondrial preparations to provide insight into how changes in mitochondrial quantity and quality contribute to variation in the respiratory capacity of tissues. Mitochondrial preparations from permeabilized muscle fibres can be used to

measure the full respiratory capacity of muscle tissue. This can be combined with indices of mitochondrial abundance, along with histological measurements of muscle fibre-type composition, to discern the mechanisms for variation in respiratory capacity. For example, expressing mitochondrial respiration relative to CS activity provides an index of mitochondrial quality that can be compared between treatments/species/etc. (Jacobs and Lundby 2013; MacInnis et al. 2017). Preparations of mitochondria isolated from muscle tissue can also be used to directly examine whether variation in mitochondrial quality might contribute to variation in the respiratory capacity of muscle tissue, because the amount of mitochondria under study can be controlled (e.g., respiration can be measured for a set amount of mitochondrial protein). This approach must be used with some caution, in consideration of the possibility that the function of isolated mitochondria may not always reflect the function of mitochondria *in situ* (see above), but it does offer several advantages. For example, it allows for the separate isolation and functional characterization of distinct subpopulations of subsarcolemmal (the subpopulation located directly adjacent to the cell membrane) and intermyofibrillar (the subpopulation situated between myofibrils) mitochondria (Koves et al. 2005), and it can also be used to examine how mitochondrial respiration is affected by hypoxia (Gnaiger et al. 1998; Scott et al. 2009a; Larsen et al. 2011), which is not possible in permeabilized muscle fibres due to significant O_2 diffusion limitation.

Evolution of mitochondrial respiratory capacity in high-altitude natives

The aerobic performance of organisms is supported by the integrated function of several tissues, many of which must support high rates of mitochondrial respiration at an organism's

aerobic capacity. Aerobic exercise and thermogenesis both require high rates of respiration in skeletal muscles to support the energy demands of muscle movement and shivering, respectively. These activities also require the skeletal muscles that support breathing (e.g., the diaphragm in mammals) and the cardiac muscle in the heart to sustain elevated rates of respiration to support cardiorespiratory O_2 transport. In this section, we will examine how variation in the respiratory capacity of each of these tissues contributes to adaptive variation in aerobic performance by reviewing our work on high-altitude deer mice. In doing so, we will explore the relative contributions of mitochondrial quantity and quality to variation in tissue respiratory capacity in high-altitude natives.

Evolved changes in the locomotory muscles of high-altitude natives

High-altitude deer mice have evolved a greater mitochondrial respiratory capacity in the gastrocnemius muscle compared to their low-altitude counterparts (Fig. 2A) (Mahalingam et al. 2017), in concert with the evolved population differences in VO_2max in hypoxia (Cheviron et al. 2013; Lui et al. 2015; Tate et al. 2017). This arises from an overall increase in OXPHOS capacity in highlanders compared to lowlanders, but this capacity is relatively unaffected by hypoxia acclimation. The gastrocnemius is a large muscle of mixed fibre-type composition in the lower hindlimb, and is used for both locomotion and shivering (Günther et al. 1983; Pearson et al. 2005), so this tissue should represent an important site of O_2 demand at VO_2max. In contrast, there is no comparable variation in the respiratory capacity of the soleus (Mahalingam et al. 2017), a smaller hindlimb muscle that is already highly oxidative and plays a more important role in posture and slow movements (Nicolopoulos-Stournaras and Iles 1984).

Evolved increases in mitochondrial quantity appear to be the predominant cause of the increases in respiratory capacity in the gastrocnemius. High-altitude mice have a greater proportional abundance of oxidative fibre-types in this muscle (Lui et al. 2015; Scott et al. 2015a), which would increase the mitochondrial abundance and respiratory capacity of a given mass/volume of tissue. However, this appears to be caused by a reduction in the total number of glycolytic fibres in the gastrocnemius, rather than an increase in the total number of oxidative fibres, coincident with a reduction in gastrocnemius mass (Mahalingam et al., unpublished). Furthermore, the magnitude of the population difference in the density of oxidative fibres in the muscle (25-30% by volume) is less than the population difference in respiratory capacity (40-50% by mass; Fig. 2A) (Mahalingam et al. 2017). This discrepancy can be explained by our observation that mitochondrial volume density in oxidative fibres is 25% greater in highlanders than in lowlanders (Mahalingam et al. 2017). None of these traits are affected by hypoxia acclimation in adults (Lui et al. 2015; Mahalingam et al. 2017), and they also appear to be unaffected by developmental or parental exposure to hypoxia (Nikel et al. 2017). Therefore, increases in mitochondrial quantity arise from two mechanisms – an increase in the relative density of oxidative (mitochondria-rich) fibre types, and an increase in the abundance of mitochondria within oxidative fibres – and these mechanisms exhibit little plasticity in response to chronic hypoxia.

Increases in mitochondrial abundance within the oxidative fibres of the gastrocnemius of high-altitude deer mice are entirely explained by an enrichment of subsarcolemmal mitochondria (Fig. 3) (Mahalingam et al. 2017). This strategy has the dual advantages of augmenting the respiratory capacity of the tissue while also placing more mitochondria adjacent to the cell membrane and close to the source of O_2 supply from capillaries, which should reduce

intracellular distance for O_2 diffusion. The potential benefit of this strategy for improving mitochondrial O_2 supply is emphasized by human studies showing that training-induced increases in aerobic performance are associated with a preferential proliferation of subsarcolemmal mitochondria (Hoppeler et al. 1985). This strategy could be especially advantageous in hypoxia, when circulatory O_2 supply is at a premium.

This preferential proliferation of subsarcolemmal mitochondria in high-altitude deer mice raises the intriguing question of whether mitochondrial distribution is subject to a trade-off between mitochondrial O_2 supply and intracellular ATP transport. Subsarcolemmal mitochondria are further than intermyofibrillar mitochondria from some of the key sites of ATP demand in the muscle fibre (e.g., myofibrillar ATPase), so it is possible that intracellular diffusion distances are greater for the ATP produced by subsarcolemmal mitochondria. However, this possibility may be precluded by the fact that the mitochondria within muscle fibres form a complex reticulum, in which there are pervasive interconnections between the subsarcolemmal and intermyofibrillar subpopulations (Bakeeva et al. 1978; Kayar et al. 1988). The mitochondrial reticulum is believed to allow for a distribution of labour between the subpopulations, in which the subsarcolemmal mitochondria are specialized for consuming oxygen and generating the proton-motive force, and the intermyofibrillar mitochondria use the proton-motive force for ATP production (Glancy et al. 2015). Oxidative fibres in the gastrocnemius of highland deer mice have a similar abundance of intermyofibrillar mitochondria to lowland deer mice, so it is possible that their capacity to produce ATP close to the sites of ATP demand is maintained, while their greater abundance of subsarcolemmal mitochondria augments their ability to take up and consume oxygen and generate the proton-motive force needed for ATP synthesis. High-altitude deer mice also express elevated levels of mitochondrial creatine kinase in the gastrocnemius (Scott et al. 2015a), which

could enhance the intracellular shuttling of ATP *via* the phosphocreatine shuttle (Ventura-Clapier et al. 1998).

The evolved changes in the gastrocnemius muscle of high-altitude deer mice appear to be underpinned by integrated changes in gene expression. Population differences in oxidative capacity and fibre-type composition are associated with population differences in the expression of several genes involved in energy metabolism, muscle development, and vascular development (Cheviron et al. 2014; Scott et al. 2015a). For example, highlanders had elevated transcript levels for several enzymes involved in OXPHOS and β-oxidation of lipids, mitochondrial creatine kinase (as discussed above), and mitochondrial ribosome proteins (Cheviron et al. 2014; Scott et al. 2015a). Highlanders also have elevated transcript and protein abundances of the nuclear receptor PPARγ (peroxisome proliferator-activated receptor gamma) (Lui et al. 2015), which is well known to regulate lipid metabolism and may contribute to the regulation of mitochondrial biogenesis in skeletal muscle (Amin et al. 2010).

The potential adaptive value of evolved changes in muscle phenotypes is underscored by the convergent evolution of these traits in the locomotory muscle of multiple high-altitude lineages. The bar-headed goose flies at high altitudes during its migration across the Himalayas (Hawkes et al. 2013; Scott et al. 2015b), and therefore has a similar need to high-altitude deer mice for maintaining aerobic performance and high rates of mitochondrial respiration during hypoxia. Bar-headed geese have a greater abundance of oxidative fibres in the pectoralis (the primary muscle used for flapping flight) than low-altitude (but still strong-flying) species of geese, and they also have a greater proportion of their mitochondria in a subsarcolemmal location (Fig. 3) (Scott et al. 2009a). Bar-headed geese may also have a more active phosphocreatine shuttle in pectoralis muscle fibres, based on observations that creatine

sensitivity of mitochondrial respiration is enhanced in this species (Scott et al. 2009b). Therefore, despite the many differences between mice and geese, and the different patterns of hypoxia experienced by high-altitude residents versus migrants, they appear to use some similar mechanisms for augmenting muscle respiration and aerobic performance in hypoxia. Some evidence in human Sherpa populations suggests that the evolved changes in high-altitude deer mice may not occur in all other high-altitude natives (Kayser et al. 1991; Horscroft et al. 2017), but it has often been difficult in human studies to distinguish whether high-altitude phenotypes arise from evolved (genetically-based) adaptations or from effects of developing in a hypoxic environment (Brutsaert 2016; Moore 2017).

The importance of changes in mitochondrial quality to the increased respiratory capacity in the gastrocnemius muscle of high-altitude deer mice is unclear. The findings described above from measurements of permeabilized fibre respiration, fibre-type composition, and mitochondrial volume density suggest that evolved increases in mitochondrial quantity lead to population differences in tissue respiratory capacity that are relatively unaffected by environmental hypoxia. However, we have found that the cristae surface density of mitochondria increases in both populations after hypoxia acclimation (Mahalingam et al. 2017). This might be expected to increase the specific respiratory capacity of a given volume of mitochondria if it also augments the density of ETS complexes (Nielsen et al. 2017), although variation in cristae surface density may not always be associated with comparable variation in mitochondrial respiration (Suarez et al. 1991), and cristae morphology can vary for other reasons (e.g., structural organization of enzyme complexes in the membrane) (Davies et al. 2011). We have also observed there to be variation in the respiratory capacity of mitochondria isolated from the hindlimb muscle that is not concordant with the variation observed in permeabilized

gastrocnemius fibres. Specifically, we have found that mitochondrial respiration is greater in highlanders than in lowlanders when populations are compared in normoxia, but not when compared after hypoxia acclimation, because mitochondrial respiration increases in lowlanders to the high but non-plastic respiration rates exhibited by highlanders (Mahalingam et al. 2017). The reason for this discordance between mitochondrial preparations is not clear. It is possible that there were differences in mitochondrial function between the tissues used for muscle fibre respiration (gastrocnemius) and those used for mitochondrial isolation (the entirety of all hindlimb muscles, not just the gastrocnemius). It is also possible that there were changes in mitochondrial physiology during the isolation process (see above). Nevertheless, the evidence suggests that there is variation in mitochondrial quality between high- and low-altitude populations of deer mice, but the importance of this variation for muscle respiratory capacity and aerobic performance in hypoxia remains to be fully appreciated.

Evolution of phenotypic plasticity in the respiratory muscle of high-altitude natives

Hypoxia acclimation augments the mitochondrial respiratory capacity of the diaphragm muscle in *Peromyscus* mice (Fig. 2B) (Dawson et al. 2018), which may contribute to the phenotypic plasticity of aerobic performance and/or respiratory function in chronic hypoxia (Lui et al. 2015; Ivy and Scott 2017; Tate et al. 2017). This increase in the overall OXPHOS capacity of the diaphragm could represent a training response to increases in muscle activity, because breathing increases 1.2- to 1.5-fold at these levels of hypoxia (Ivy and Scott 2017). However, although hypoxia acclimation increases mitochondrial respiratory capacity in both populations, deeper levels of hypoxia are needed to elicit a response in high-altitude mice (Fig. 2B) (Dawson et al. 2018).

High-altitude deer mice also differ in the relative importance of mitochondrial quantity *versus* mitochondrial quality for increasing respiratory capacity. The increases in mitochondrial respiratory capacity in the diaphragm of lowlanders appeared to be caused by increases in both mitochondrial abundance and the specific OXPHOS capacity of the mitochondria, because there were increases in both CS activity and respiration relative to CS activity in the tissue (Dawson et al. 2018). In contrast, the increases in diaphragm respiratory capacity in highlanders appeared to be caused by increases in tissue mitochondrial abundance, because respiration relative to CS activity did not change after acclimation to moderate (12 kPa O_2) or more severe (9 kPa O_2) levels of hypoxia (Dawson et al. 2018). In neither case were there any changes in muscle fibre-type composition (nor were there any population differences), so the increases in tissue respiratory capacity were entirely attributable to changes in mitochondrial quality/quantity within particular fibre types.

The population differences in the effects of chronic hypoxia on mitochondrial respiratory capacity suggest that phenotypic plasticity of the diaphragm has evolved in high-altitude deer mice. The pattern of variation represents counter-gradient variation, a term that describes situations in which evolved (genetically based) variation in a trait oppose the effects of phenotypic plasticity, and thereby act to minimize phenotypic change along an environmental gradient (Conover and Schultz 1995). This could have arisen as a secondary indirect consequence of an evolved change that reduced the stimulus for diaphragm plasticity in high-altitude mice. For example, chronic hypoxia has different effects on breathing and the magnitude of arterial hypoxaemia between populations, which could have influenced diaphragm activity and plasticity in chronic hypoxia (Ivy and Scott 2017). Alternatively, the effects of high-altitude adaptation could have been more direct, acting to blunt diaphragm plasticity specifically. This

might occur if increases in diaphragm respiratory capacity are non-adaptive (or even maladaptive) at high altitudes (Ghalambor et al. 2007). The reason why this might be the case is unclear, but examples of counter-gradient variation can often be explained on the basis of trade-offs between the trait of interest and other traits that affect fitness (Conover and Schultz 1995).

Lack of variation in the cardiac muscle of high-altitude natives

We have examined the mitochondrial respiratory capacity of the cardiac muscle in deer mice using similar approaches to those employed in the studies described above for skeletal muscles. These data have not been published previously, but they were acquired using the same captive breeding populations that we have established in our previous studies (Tate et al. 2017), and mitochondrial respiration was measured in permeabilized fibres from the left ventricle using very similar protocols to those we have used in published studies of gastrocnemius, soleus, and diaphragm muscles (Mahalingam et al. 2017; Dawson et al. 2018). A detailed description of the methods used can be found in the Supplementary Materials.

Unlike the variation exhibited in locomotory and respiratory muscles, high-altitude adaptation does not appear to have affected mitochondrial respiratory capacity in the heart of deer mice. Respiration of left ventricle fibres was unaffected by hypoxia acclimation and varied little between populations (Fig. 4). Highlanders had a modestly lower respiratory capacity for pyruvate oxidation (P_{PM}), as reflected by a significant main effect of population in two-factor ANOVA ($p=0.043$), but this was not a result of any differences in OXPHOS capacity *via* complex I, complexes I and II combined, or complex IV. This result is consistent with some previous findings, in which variation in whole-animal aerobic performance between species/populations or in response to exercise training was not associated with changes in the

mitochondrial abundance or distribution within heart muscle fibres (Kayar et al. 1986; Kayar et al. 1989; Conley et al. 1995). However, high-altitude mice appear capable of sustaining higher heart rates and possibly stroke volumes that contributes their greater VO_2max in hypoxia (Tate et al. 2017). Our results here suggest that this does not result from variation in mitochondrial respiratory capacity, but may instead arise from population differences in O_2 supply to cardiac tissue or in some other aspect of cardiac function.

Mitochondrial production of reactive oxygen species in high-altitude natives

Mitochondria play a critical role in reactive oxygen species (ROS) homeostasis, which raises the intriguing question of whether evolved variation in this aspect of mitochondrial physiology contributes to adaptive variation in aerobic performance, or whether it contributes to high-altitude adaptation in some other way. Mitochondria are a primary source of ROS in the cell, and *in vitro* studies suggest that roughly 0.1-2% of mitochondrial O_2 consumption ends in ROS formation rather than being consumed by cytochrome c oxidase (Murphy 2009). Acute exercise increases mitochondrial ROS production (Pearson et al. 2014), which has long been known to induce a transient state of oxidative stress in the muscle (Fisher-Wellman and Bloomer 2009), but is also a critical signal that induces many of the beneficial cellular and molecular responses of skeletal muscle to training (Mason et al. 2016). ROS release from muscle mitochondria later declines after prolonged exercise training (Venditti et al. 1999; Gram et al. 2015), coincident with a rise in the activity and/or expression of some antioxidant enzymes in muscle tissue (Gore et al. 1998; Lambertucci et al. 2007). Increases in the capacity of skeletal muscle for respiration and heat production in response to prolonged cold exposure can also be associated with changes in mitochondrial quality that reduce mitochondrial ROS emission (e.g.,

expression of uncoupling proteins) (Rey et al. 2010). Some (though not all) evidence also suggests that chronic hypoxia can cause oxidative stress under some conditions (Dosek et al. 2007; Aon et al. 2010), which could provide a benefit for adaptive changes in mitochondrial ROS emission (Du et al. 2016; Mahalingam et al. 2017). Therefore, it is foreseeable that high-altitude natives could alter mitochondrial ROS physiology to alter cell signalling or reduce oxidative stress in muscle tissues during chronic hypoxia and/or cold exposure.

Is ROS production by muscle mitochondria altered in deer mice native to high altitudes? Yes appears to be the answer to this question, but the mechanisms involved likely differ between muscles. Hypoxia acclimation reduces ROS emission from mitochondria isolated from the hind limb muscles, in a manner that is not clearly related to variation in mitochondrial respiration or tissue respiratory capacity and that does not differ between high- and low-altitude populations (Mahalingam et al. 2017). In contrast, hypoxia acclimation augments ROS emission from diaphragm mitochondria in low-altitude deer mice (Dawson et al. 2018). This difference between muscles may arise from the stark differences in how they respond to chronic hypoxia – the activity of locomotory muscles is likely unchanged or even reduced in chronic hypoxia, whereas the activity of diaphragm muscle likely increases due to stimulation of breathing by hypoxia. However, hypoxia acclimation has no effect on ROS emission from diaphragm mitochondria in high-altitude deer mice, such that highlanders have lower mitochondrial ROS emission than lowlanders in hypoxia (Dawson et al. 2018). The mechanisms accounting for this evolved reduction in ROS release are unclear, but could be related to the divergent mechanisms used to increase mitochondrial respiratory capacity in highlanders versus lowlanders during hypoxia acclimation (see above). Evidence in highland human populations (Tibetans and Sherpas) suggests that evolved improvements in ROS mitigation may be important for reducing the

prevalence of oxidative stress in the muscle at high altitude (Gelfi et al. 2004; Horscroft et al. 2017). Whether the evolved change in mitochondrial ROS emission in high-altitude deer mice also serves to avoid oxidative stress, or whether it instead acts to alter ROS-mediated signalling (Veal et al. 2007) or is a secondary consequence of mitochondrial restructuring for another purpose, has yet to be determined.

Conclusions

Evolved changes in mitochondrial quantity and quality in skeletal muscles appear to make important contributions to adaptive variation in aerobic performance in deer mice. High-altitude populations have evolved a higher VO_2max in hypoxia than low-altitude populations, overlaid upon increases in VO_2max that occur in response to hypoxia acclimation. The evolved differences in VO_2max are associated with comparable differences in the respiratory capacity of the gastrocnemius muscle, a tissue that is likely a key ATP consumer during aerobic exercise and thermogenesis. This is primarily attributable to evolved increases in mitochondrial quantity in high-altitude mice, arising from a higher proportional abundance of oxidative fibre types and a greater volume density of subsarcolemmal mitochondria within oxidative fibres. Hypoxia acclimation increases the respiratory capacity of the diaphragm but the mechanisms responsible for these particular increases appeared to differ between populations, involving changes in both mitochondrial quantity and quality in lowlanders, but only mitochondrial quantity in highlanders. Therefore, our results suggest that evolutionary changes in mitochondrial quantity and quality can represent an important mechanism underlying the evolution of complex physiological traits.

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Figure Legends

Fig. 1 Representative measurements of mitochondrial respiration (normalized to mitochondrial protein content) for mitochondria isolated from the gastrocnemius muscle (A) or heart ventricle (B) of an individual deer mouse (*Peromyscus maniculatus*). Thick lines represent traces of OXPHOS respiration in well-coupled mitochondria (solid lines), or respiration in the presence of the protonophore CCCP (carbonyl cyanide m-chlorophenyl hydrazone; 0.25 μM) (dashed lines). CCCP collapses the proton gradient and uncouples electron transport from ATP synthesis, and can thus be used to measure the full respiratory capacity of the electron transport system (ETS). Thin dashed lines indicate the points at which substrate/uncoupler/inhibitor were added. Mitochondrial respiration is low under non-phosphorylating conditions in the presence of malate (2 mM) and pyruvate (5 mM), which reflects respiration and electron transport that opposes proton leak. ADP (5 mM) stimulates respiration, but respiration with only malate and pyruvate may still be limited by the capacity for pyruvate transport and oxidation. Subsequent addition of glutamate (10 mM) then succinate (10 mM) is thought to stimulate the full respiratory capacity for OXPHOS or of the ETS, with electron entry *via* complex I and then both complexes I and II. Inhibition of complex I with rotenone (0.5 μM) stimulates respiration *via* complex II alone. Finally, addition of ascorbate (2 mM) followed by TMPD (N,N,N′,N′- tetramethyl-p-phenylenediamine; 0.5 mM) stimulates respiration with electron input directly to complex IV. Mitochondrial isolation and respirometry methods were otherwise similar to those used in our previous work (Mahalingam et al. 2017).

Fig. 2 Capacities for mitochondrial respiration in permeabilized fibres from locomotory muscle (gastrocnemius) and respiratory muscle (diaphragm) in high-altitude and low-altitude populations of *Peromyscus* mice. The respiration data shown reflect the capacities for oxidative phosphorylation with substrates that result in electron entry *via* complexes I and II of the electron transport system (malate, pyruvate, glutamate, succinate, ADP), and the pattern of variation is representative of similar variation we have observed using other substrate combinations. [*,†] Significant pairwise differences between populations within an environment, or between environments within a population, respectively. Data are reproduced with permission (Mahalingam et al. 2017; Dawson et al. 2018).

Fig. 3 High-altitude natives have a greater abundance of subsarcolemmal mitochondria in locomotory muscle. Transmission electron microscopy (TEM) images from the oxidative core of the gastrocnemius muscle in deer mice from high-altitude (A) and low-altitude (B) populations, and from the pectoralis flight muscle of high-altitude bar-headed geese (C) and low-altitude barnacle geese (D), all raised in common garden conditions at sea level. Scale bars each represent 10 μm (A and B are at the same magnification, and C and D are at the same magnification). Arrow, subsarcolemmal mitochondria; arrowhead, intermyofibrillar mitochondria; c, capillary; mf, myofibrils. Images are reproduced with permission (Scott et al. 2009a; Mahalingam et al. 2017).

Fig. 4 The respiratory capacities of permeabilized fibres from the left ventricle are similar in high-altitude and low-altitude populations of deer mice. Leak respiration was measured in the absence of adenylates (L_N; with malate and pyruvate), and oxidative phosphorylation (P) was

measured with substrates that result in electron entry *via* complex I (P_{PM}: malate, pyruvate, ADP; P_{PMG}: malate, pyruvate, glutamate, ADP), complexes I and II (P_{PMGS}: malate, pyruvate, glutamate, succinate, ADP), or complex IV (P_{Tm}: ADP, ascorbate, TMPD). N, normoxia acclimation; H, hypoxia acclimation. Data are presented as means ± SEM (n = 9 for hypoxia-acclimated individuals, n = 10 for all other groups), and were compared statistically using two-factor ANOVA. Main effects of population: L_N, $F_{1,35}$=0.50, p=0.483; P_{PM}, $F_{1,35}$=4.42, p=0.043; P_{PMG}, $F_{1,35}$=0.19, p=0.665; P_{PMGS}, $F_{1,35}$=0.06, p=0.809; P_{Tm}, $F_{1,35}$=0.33, p=0.571. Main effects of hypoxia acclimation: L_N, $F_{1,35}$=0.40, p=0.533; P_{PM}, $F_{1,35}$=0.002, p=0.963; P_{PMG}, $F_{1,35}$=0.15, p=0.700; P_{PMGS}, $F_{1,35}$=0.04, p=0.851; P_{Tm}, $F_{1,34}$=0.15, p=0.705. There were no statistically significant interactions. There were also no significant pairwise differences between populations, or in response to hypoxia acclimation within a population (P>0.05).

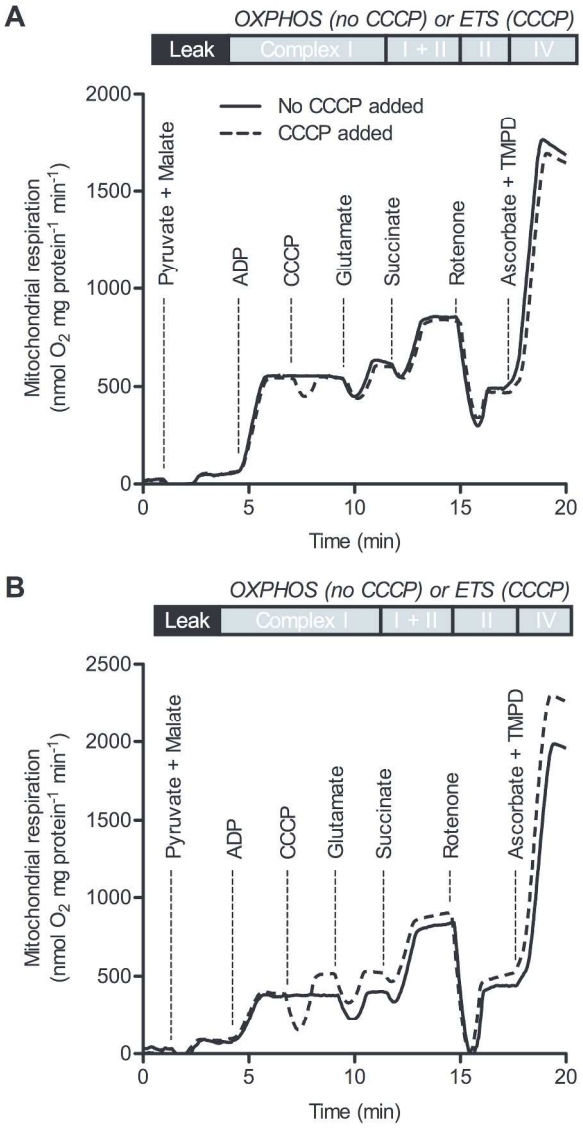

Fig. 1 Representative measurements of mitochondrial respiration (normalized to mitochondrial protein content) for mitochondria isolated from the gastrocnemius muscle (A) or heart ventricle (B) of the deer mouse (*Peromyscus maniculatus*).

176x310mm (300 x 300 DPI)

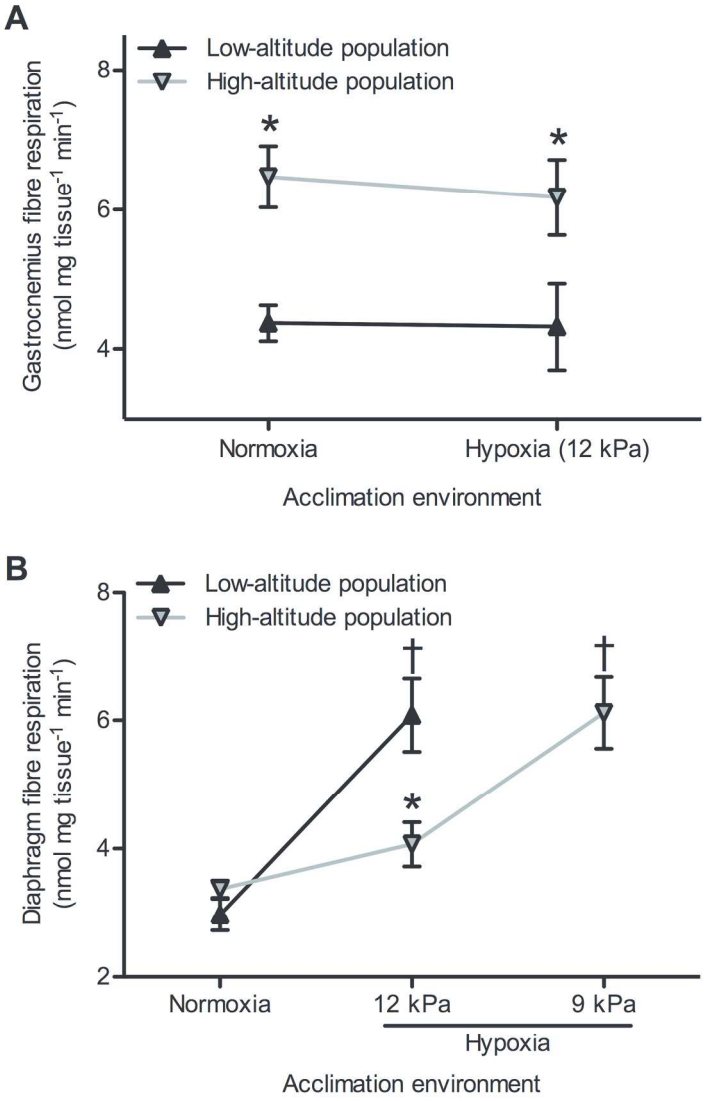

Fig. 2 Capacities for mitochondrial respiration in permeabilized fibres from locomotory muscle (gastrocnemius) and respiratory muscle (diaphragm) in high-altitude and low-altitude populations of *Peromyscus* mice.

152x216mm (300 x 300 DPI)

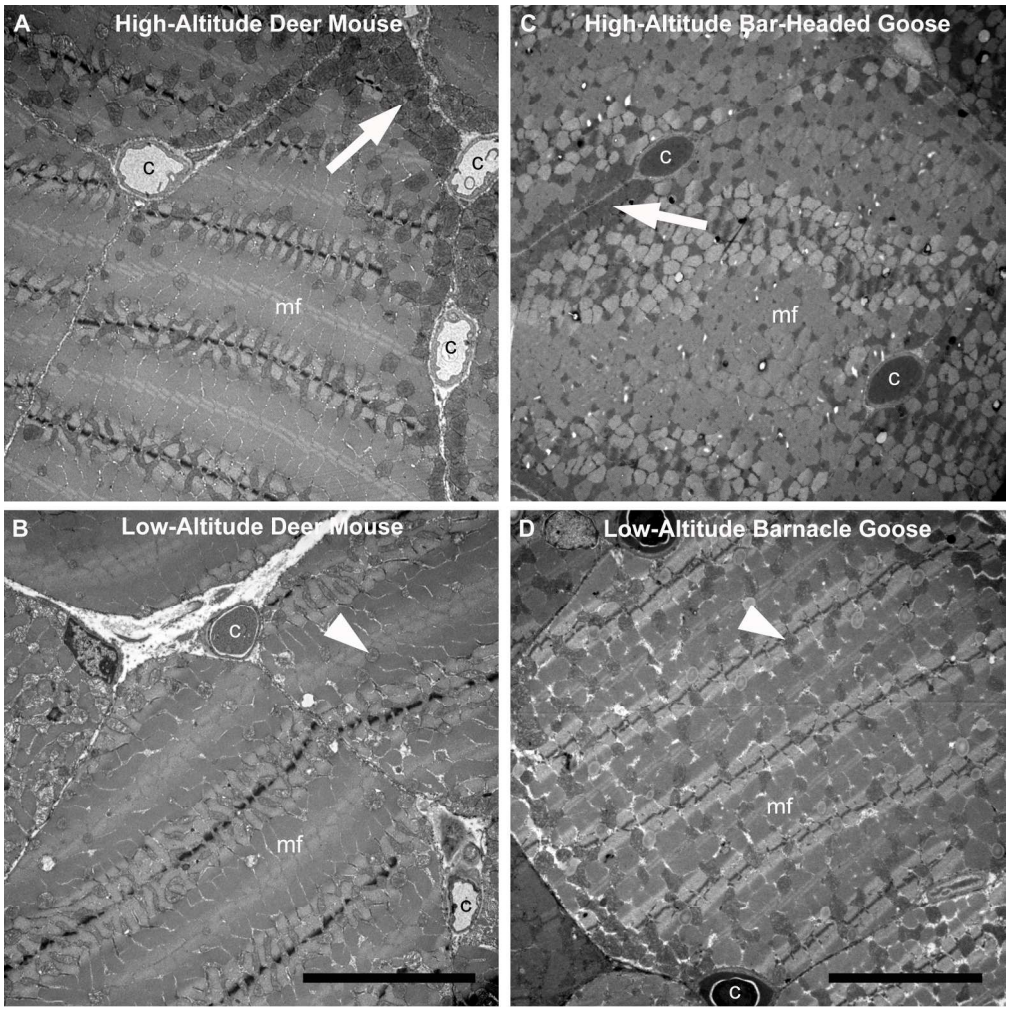

Fig. 3 High-altitude natives have a greater abundance of subsarcolemmal mitochondria in locomotory muscle.

180x179mm (300 x 300 DPI)

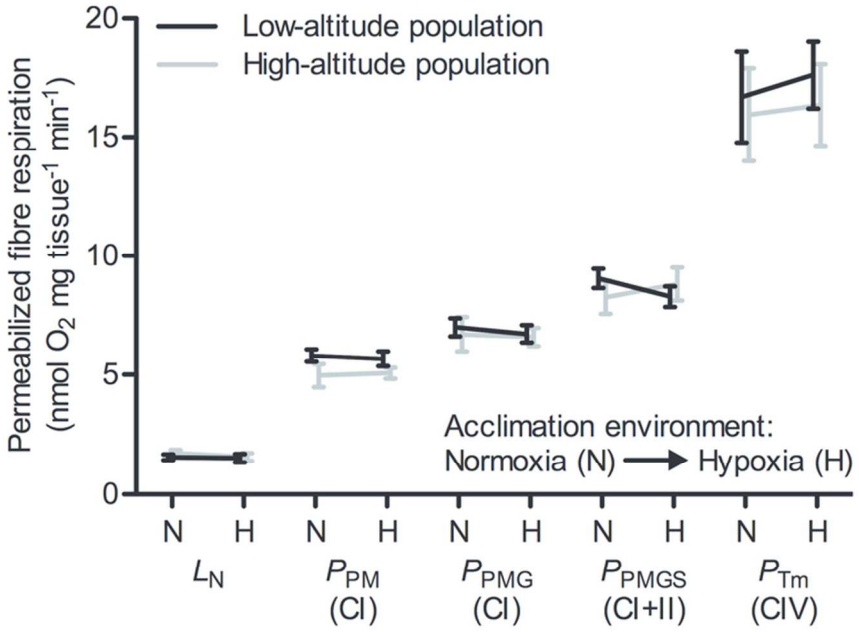

Fig. 4 The respiratory capacities of permeabilized fibres from the left ventricle are similar in high-altitude and low-altitude populations of deer mice.

76x55mm (300 x 300 DPI)

SUPPLEMENTARY MATERIAL

This section provides a detailed materials and methods section that describes how we measured the mitochondrial respiratory capacity of cardiac muscle from the left ventricle of deer mice.

Materials and Methods

We carried out this work using the captive breeding populations we have established in our previous studies (Tate et al. 2017). Breeding populations were created from deer mice that were live trapped at high altitude near the summit of Mount Evans in Colorado (4350 m above sea level) (*P. m. rufinus*) and at low altitude in the Great Plains of Nebraska (~400 m above sea level) (*P. m. nebracensis*). Wild adult mice were housed in common lab conditions, and were bred within each population to produce lab-raised progeny with highland and lowland ancestry. Second generation mice were raised in standard holding conditions (24-25°C, 12 h light: 12 h dark photoperiod) with unlimited access to chow and water until they reached 6 months of age. Mice from each population were then acclimated to either (i) normobaria in standard normoxic holding conditions (n = 9), or (ii) hypobaric hypoxia (barometric pressure of 60 kPa and O_2 pressure ~12 kPa; equivalent to those at an elevation of ~4300 m) (n = 10) in specially designed hypobaric chambers that have been described previously (McClelland et al. 1998; Lui et al. 2015). Cages were cleaned twice a week during acclimations, which required that the hypobaric groups be returned to normobaria for a brief period (<30 min). Mice were euthanized (using an overdose of isoflurane followed by cervical dislocation) and sampled after 6-8 weeks of acclimation. All animal protocols followed guidelines established by the Canadian Council on Animal Care and were approved by the McMaster University Animal Research Ethics Board.

Mitochondrial respiration was measured for permeabilized fibres from the left ventricle of the heart, using a very similar protocol to those we have used previously (Mahalingam et al. 2017; Dawson et al. 2018). Small samples of the left ventricle were placed in ice-cold relaxing biopsy preservation solution (BIOPS, in mM: 2.77 CaK_2EGTA, 7.23 K_2EGTA, 5.77 Na_2ATP, 6.56 $MgCl_2$, 20 taurine, 15 Na_2Phosphocreatine, 20 imidazole, 0.5 DTT, 50 MES; pH 7.1). The muscle was mechanically separated using dissecting probes, chemically permeabilized for 30 min in BIOPS containing 52.5 mg/l saponin, and washed three times for 10 min in respiration buffer (MiR05, in mM: 0.5 EGTA, 3 $MgCl_2$, 60 K-lactobionate, 20 taurine, 10 KH_2PO_4, 20

HEPES, 110 sucrose, 1 g/l fatty-acid free bovine serum albumin; pH 7.1) to wash out endogenous adenine nucleotides and other substrates. Muscle fibres were weighed and then transferred to 2 ml of MiR05 in a high-resolution respirometer (Oxygraph-O2k, Oroboros Instruments), maintained at 37°C under continuous stirring. Pyruvate (10 mM) and malate (2 mM) were added to stimulate leak state respiration in absence of ADP. Oxidative phosphorylation was stimulated with the addition of 6 mM ADP. Cytochrome c (10 μM) was added to assess the integrity of the outer mitochondrial membrane (increases in respiration in response to exogenous cytochrome c are often used as an index of poor membrane integrity, but none of our preparations showed cytochrome c effect of more than 5%). Glutamate (25 mM) and then succinate (10 mM) were added to elicit maximal respiration *via* complex I and complexes I+II, respectively. Ascorbate (2 mM), TMPD (0.5 mM), and antimycin A (12.5 μM) were added to inhibit complex III and stimulate maximal respiration *via* complex IV. We waited ~2 min after each addition until a stable respiration rate was achieved before proceeding, and all measurements were made above O_2 concentrations of 200 μM to eliminate possible effects of O_2 limitation. The measured respiration rates were corrected for background O_2 flux of the O_2 sensor (Gnaiger et al. 1995) and are expressed relative to muscle fibre weight. Two-factor ANOVA and Bonferroni multiple-comparisons post-tests were used as appropriate to determine the main effects of population altitude, acclimation environment, and their interaction.

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