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Author/s:

Lynch, GS;Koopman, R

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Overcoming nature's paradox in skeletal muscle to optimise animal production

Gordon S. Lynch ^{A,B} and René Koopman^A

^ACentre for Muscle Research, Department of Physiology, The University of Melbourne, Vic. 3010, Australia.

^BCorresponding author. Email: gsl@unimelb.edu.au

Abstract. Nature's paradox in skeletal muscle describes the seemingly mutually exclusive relationship between muscle fibre size and oxidative capacity. In mammals, there is a constraint on the size at which mitochondria-rich, high O₂-dependent oxidative fibres can attain before they become anoxic or adapt to a glycolytic phenotype, being less reliant on O₂. This implies that a muscle fibre can hypertrophy at the expense of its endurance capacity. Adaptations to activity (exercise) generally obey this relationship, with optimal muscle endurance generally being linked to an enhanced proportion of small, slow oxidative fibres and muscle strength (force and/or power) being linked to an enhanced proportion of large, fast glycolytic fibres. This relationship generally constrains not only the physiological limits of performance (e.g. speed and endurance), but also the capacity to manipulate muscle attributes such as fibre size and composition, with important relevance to the livestock and aquaculture industries for producing specific muscle traits such as (flesh) quality, texture and taste. Highly glycolytic (white) muscles have different traits than do highly oxidative (red) muscles and so the ability to manipulate muscle attributes to produce flesh with specific traits has important implications for optimising meat production and quality. Understanding the biological regulation of muscle size, and phenotype and the capacity to manipulate signalling pathways to produce specific attributes, has important implications for promoting ethically sustainable and profitable commercial livestock and aquaculture practices and for developing alternative food sources, including 'laboratory meat' or 'clean meat'. This review describes the exciting potential of manipulating muscle attributes relevant to animal production, through traditional nutritional and pharmacological approaches and through viral-mediated strategies that could theoretically push the limits of muscle fibre growth, adaptation and plasticity.

Additional keywords: muscle fibre, muscle fiber, muscle growth, muscle plasticity, oxidative, glycolytic.

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Introduction

Skeletal muscles are remarkably plastic, capable of modifying their phenotype according to functional demand and perturbations in innervation, load, hormones and other regulating factors. Muscle fibres can be defined physiologically by their speed of contraction and resistance to fatigue, which are properties that are determined by contractile and regulatory protein isoforms and metabolism. Quickly contracting muscles are composed predominantly of fast (myosin) isoforms; slowly contracting muscles are composed of slow (myosin) isoforms. Metabolic properties provide the capacity for sustained contraction, with muscles contracting forcefully but infrequently relying on anaerobic metabolism, and more frequently contracting muscles relying on oxidative metabolism (Pette 2001; Schiaffino 2007; Schiaffino and Reggiani 2011; Lynch 2017).

While skeletal muscles can adapt to imposed demands, there are physiological constraints on muscle fibre size and composition that ultimately limit adaptation. An intriguing and unresolved question in muscle biology is what governs and limits adaptation and plasticity, which is often described

as the 'muscle paradox' (van Wessel *et al.* 2010). This implies that muscle fibres challenged to simultaneously increase their size/mass/strength (hypertrophy) and fatigue resistance (oxidative capacity) will increase strength or fatigue resistance to a lesser extent than do fibres increasing either of these attributes alone (Fig. 1; van der Laarse *et al.* 1998; van Wessel *et al.* 2010; van der Zwaard *et al.* 2018). Adaptations to activity (exercise) generally obey this relationship, with optimal muscle endurance being generally linked to an enhanced proportion of small, slow oxidative fibres, and muscle strength (force and/or power) being linked to an enhanced proportion of large, fast glycolytic fibres. The paradox has (until now) constrained not only the physiological limits of performance (e.g. speed and endurance), but also the capacity to manipulate muscle attributes such as fibre size and composition, with potential application to the livestock and aquaculture industries for producing specific muscle traits relevant to flesh quality, texture and taste. Muscle quality and quantity are strongly influenced by environmental factors (e.g. nutrition and exercise) and manipulating pathways regulating muscle protein and fat composition can alter texture and fat

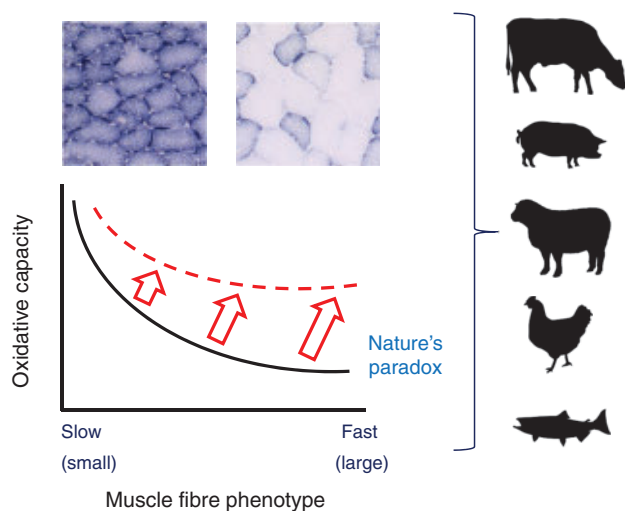


Fig. 1. 'Nature's paradox' for skeletal muscle (solid line) suggests that fibre size and oxidative capacity are mutually exclusive, thus limiting adaptation. The fibres with high oxidative capacity tend to be small and slow contracting (inset, left panel). Highly glycolytic (pale) muscle fibres tend to be large and fast contracting (inset, right panel). Overcoming the paradox (dashed line) to create larger, more oxidative muscle fibres through genetic, nutritional or pharmacological interventions could enhance muscle attributes (such as fibre size and muscle phenotype), with important implications for animal production in livestock (e.g. beef, lamb, pork and poultry) and aquaculture (e.g. salmon) industries. Targeting and manipulating specific muscle attributes in this context could ultimately affect parameters such as feed efficiency and yield, as well as product (flesh) quality, colour and texture, relevant to domestic and export market preferences.

deposition. Genetic selection to promote muscle size has been negatively correlated with textural quality in fish and livestock (Chen and Lee 2016; Moghadam *et al.* 2017; Robinson *et al.* 2017), such that selecting for muscle growth typically leads to more glycolytic ('white'), tougher meat. The robustness of this paradoxical relationship is unclear, because sophisticated molecular tools, including viral-vector technologies, can now be applied to force expression of specific muscle attributes in ways not examined previously.

Significance of skeletal muscle for animal production

Skeletal muscle is essential for life. We need muscles to breathe, to eat and to interact with the environment. Building and maintaining healthy muscles is needed for body heat, metabolism and movement throughout life. Yet, in later life, muscles of all mammals begin to shrink and weaken, threatening independence, productivity and quality of life. Skeletal muscle is also food, with a considerable proportion of the global population relying on muscle from livestock and fish for the best sources of high-quality proteins (Lynch and Koopman 2018). Understanding how muscles develop, grow and adapt is important not just for enhancing athletic performance or improving safety and productivity in the work place, but for livestock and aquaculture production, including optimising farming practices, nutritional feeding strategies, and meat production and quality for lucrative domestic and export markets (Lee *et al.* 2010; Lefaucheur 2010; Astruc 2014; Listrat *et al.* 2016; Parr *et al.* 2016).

Skeletal muscle fibres

Muscle comprises functionally diverse fibre types ranging in size, metabolism and contractility (Burke *et al.* 1973; Larsson *et al.* 1991; Schiaffino 2007; Schiaffino and Reggiani 2011; Blaauw *et al.* 2013). Muscle phenotype is largely defined by fibre number, fibre cross-sectional area, muscle architecture and fibre-type distribution. On the basis of myosin heavy-chain protein isoforms, which largely dictate the rate of force development, velocity of shortening and rate of cross-bridge cycling, mammalian muscle fibres are broadly classified as slow-twitch (Type I) or fast-twitch (Types IIa, IIx and IIb). While Type I and Type IIa fibres primarily generate ATP via oxidative metabolism and Type IIx and IIb fibres generate energy mostly through glycolysis (Schiaffino 2007; Murgia *et al.* 2015), fibre classification based on myosin heavy-chain isoform composition does not necessarily correlate well with oxidative capacity, and variation among species and even among mammals (e.g. the absence of Type IIb fibres in humans compared with mice and rats) should also be considered (Gouspillou *et al.* 2014). However, on the basis of an extensive body of literature examining relationships between muscle fibre cross-sectional area (fibre size) and oxidative capacity, it is generally accepted that larger fibres have relatively lower oxidative capacities than do smaller fibres, regardless of the type. In fact, both Type I and Type IIa fibres have a large oxidative capacity and are usually smaller than Type IIb and Type IIx fibres (van Wessel *et al.* 2010).

Signalling pathways regulating muscle adaptation and plasticity

Muscle fibres are highly plastic and capable of altering their properties in response to contractile demand or other perturbations of signalling pathways that regulate isoform composition. The cellular, biochemical and molecular processes governing fibre identity and regulating adaptation and plasticity, are becoming clearer. The slow-muscle fibre phenotype is controlled by biochemical signalling related to protein kinase C, calcineurin–nuclear factor of activated T-cells (NFAT), AMP-activated protein kinase, estrogen-related receptor gamma, sex-determining region Y-box 6, and peroxisome proliferator-activated receptor gamma co-activator 1- α (Tong *et al.* 2009; Ljubicic *et al.* 2014; Omairi *et al.* 2016). Calcineurin–NFAT (nuclear factor of activated T-cells) signalling plays an important role in regulating fast-to-slow muscle phenotypic adaptations (Chin *et al.* 1998). Calcineurin (gene name: Ppp3ca) is a Ca^{2+} /calmodulin-dependent phosphatase that dephosphorylates NFAT, resulting in its nuclear translocation and binding to specific sequences on the promoters of target genes that induce slow oxidative muscle fibre programming (Olson and Williams 2000; Dunn *et al.* 2001; Allen and Leinwand 2002; Parsons *et al.* 2003; Stupka *et al.* 2006, 2007, 2008). As a key master regulator of slow-muscle programming and muscle oxidative capacity, manipulating calcineurin expression is one approach to interrogate the muscle paradox and selectively alter muscle fibre composition. Each of these master regulators of muscle phenotype could be similarly explored for their ability to selectively alter muscle phenotype.

Fast, glycolytic muscles are regulated by activation of Akt signalling, and removing inhibition of Akt signalling through myostatin potently induces formation of glycolytic fibres (Trendelenburg *et al.* 2009). Myostatin, a negative regulator of skeletal muscle mass that inhibits muscle-cell differentiation, requires Smad2 and Smad3 downstream of the activin receptor II (ActRII)/activin receptor-like kinase receptor complex; Sartori *et al.* 2009). Other transforming growth-factor β (TGF- β)-like molecules can also block differentiation, including TGF- β 1, growth-differentiation factor 11 (GDF-11), activins and bone morphogenetic proteins 2 and 7. These signalling pathways for muscle growth and those for muscle atrophy that ultimately regulate muscle fibre size have been described in detail elsewhere (Glass 2010; Bodine and Baehr 2014; Rom and Reznick 2016; Winbanks *et al.* 2016).

Myostatin, also known as growth/differentiation factor-8 (GDF8), is a member of the TGF- β superfamily of secreted proteins. It is highly expressed in muscle and negatively regulates proliferation. Deletion leads to a well described hypermuscular phenotype in mice, cattle and humans, with muscles being glycolytic with reduced numbers of mitochondria (McPherron and Lee 1997; McPherron *et al.* 1997; Schuelke *et al.* 2004). Myostatin inhibitors include the myostatin propeptide, 1 follistatin and the follistatin-related gene, as well as growth and differentiation factor-associated serum protein-1 (Hill *et al.* 2003). The most potent inhibitor is follistatin and its overexpression has powerful growth-promoting effects in skeletal muscle (Zheng *et al.* 2017). Follistatin is an endogenous ligand-binding partner for myostatin and activin A (a growth factor implicated in reducing muscle fibre size; Trendelenburg *et al.* 2009; Chen *et al.* 2014; Davey *et al.* 2016; Winbanks *et al.* 2016). Myostatin antagonists are being developed as therapies for muscle-wasting diseases such as Duchenne muscular dystrophy (DMD) due to their strong hypertrophic effects on skeletal muscle (Whittemore *et al.* 2003; Murphy *et al.* 2010). Strategies to engineer (and upregulate) follistatin also have potential for these conditions, by combining the hypertrophic actions of myostatin antagonism with the anti-inflammatory and anti-fibrotic effects of activin A antagonism (Rodino-Klapac *et al.* 2009; Iskenderian *et al.* 2018; Schumann *et al.* 2018). Using viral vectors to force expression of follistatin in mouse skeletal muscles can produce phenomenal 100% (and greater) increases in skeletal muscle mass (Winbanks *et al.* 2012; Sepulveda *et al.* 2015). Strategies to inhibit myostatin or overexpress follistatin for application to animal production will be discussed later in this review.

Significance of the muscle paradox

The concept of the muscle paradox and its important physiological consequences in animals have been described in detail elsewhere (van der Laarse *et al.* 1998; Kinsey *et al.* 2007; Jimenez *et al.* 2013; Omairi *et al.* 2016; van der Zwaard S *et al.* 2018). In mammals, the paradox represents a constraint on the size at which mitochondria-rich, high O₂-dependent oxidative fibres can attain before they become anoxic or adapt to a glycolytic phenotype, less reliant on O₂. This means that a muscle fibre can hypertrophy at the expense of its endurance capacity (Fig. 1; van Wessel *et al.* 2010). Despite having an

inherent capacity to alter their attributes, the extent of change or adaptation is influenced by the number and/or magnitude of different perturbing stimuli, especially those affecting muscle fibre size and muscle fibre composition. These signalling pathways can be complementary to effect considerable change in phenotype or they may compete or interfere with each other to limit adaptations. An often-described example is that of exercising humans training simultaneously for both strength and endurance who experience less of an adaptation than if they trained just for one outcome; this is a phenomenon called the 'interference effect' or 'concurrent training effect' (Baar 2014; Fyfe *et al.* 2014; Coffey and Hawley 2017).

Typically, when muscles are loaded during resistance training, muscle fibres hypertrophy, leading to an increase in mass. Conversely, with endurance exercise, muscles adapt by increasing their oxidative metabolism facilitated through increased mitochondrial enzymes and capillary density, not through hypertrophy. The underlying mechanisms responsible for limiting adaptation (in either direction) in the face of competing stimuli remain unresolved, in part because current understanding has relied on physical activity (exercise) as the perturbing stimulus. During exercise/physical activity, only a small fraction of the total number of fibres within muscles are recruited to complete specific tasks and usually for only brief periods, such as, for example, a few seconds for maximal sprinting or powerlifting, to a few hours with endurance activities, such as marathon running. To properly interrogate the limitations of muscle adaptation and plasticity requires driving expression of key attributes using viral-vector gene-delivery tools to maximise muscle size or oxidative capacity, and so rigorously test hypotheses about the muscle paradox. Viral-vector technologies that permit direct targeting of specific growth and oxidative pathways can facilitate extremes of muscle hypertrophy and adaptive potential, with superior interrogation of the biological signalling pathways that can maximise muscle attributes singly or in combination. Overcoming nature's limits on muscle attributes would have broad application to all aspects of skeletal muscle biology, but especially to better understand muscle development and growth to optimise meat production and quality, which is relevant to animal production and livestock and aquaculture.

While viral-vector technologies provide a powerful approach for testing the limits of skeletal muscle adaptation, relevant to optimising muscle attributes from animal production, signalling pathways regulating muscle size, and phenotype can also be manipulated through nutritional and pharmacological strategies. A brief overview of selected nutritional and drug approaches for altering muscle attributes is provided.

Nutritional strategies to alter skeletal muscle attributes and phenotype

The growth of animals and their body composition (muscle, fat and bone content) can be manipulated through the energy and protein content provided in the diet. Indeed, intensive (*ad libitum*) feeding of beef cattle can improve animal growth rates, final bodyweights and feed efficiencies compared with their pasture-fed counterparts (Vestergaard *et al.* 2000a, 2000b). Here, we focus on the fundamental principles that underpin

skeletal muscle adaptation to changes in dietary intake and how these principles can be applied to enhance muscle growth and metabolism.

Protein

The most intensively studied dietary components in relation to growth of animals are protein and total energy content that can be metabolised (carbohydrate, lipids and protein). As amino acids are the building blocks for producing new protein, it is not surprising that adequate dietary protein intake is the main driver of muscle growth. Indeed, classical studies performed in growing pigs in the 1980s showed that at equivalent levels of energy intake, pigs provided a diet with adequate protein exhibited more rapid and efficient growth than did those on a protein-deficient diet (Campbell and Dunkin 1983). A more recent study in broiler chickens examined 14 different iso-energetic diets with varying macro-nutrient compositions to assess the relative importance of protein, lipid and starch on growth performance (Liu *et al.* 2017). The study confirmed that energy derived from protein was more important than non-protein energy in terms of weight gain, and that a balance between protein and energy supplies was required for efficient muscle-protein deposition (Liu *et al.* 2017). To enhance protein utilisation (nitrogen retention), digestion rate of different protein sources and amino acid composition need to be considered. For example, studies in humans have established that proteins more rapidly digested and absorbed (i.e. whey and casein hydrolysates compared with casein) result in enhanced amino acid delivery to the muscle with higher rates of protein synthesis (Koopman *et al.* 2009; Pennings *et al.* 2011).

Supplementation with animal-derived protein is more effective in stimulating protein synthesis (Tang *et al.* 2009) and promoting hypertrophy in humans than is supplementation with plant-based proteins (Wilkinson *et al.* 2007). Studies in pigs have confirmed that addition of animal-derived protein enhances performance, nutrient digestibility and gut morphology more than does addition of plant-derived protein sources (Yun *et al.* 2005). Interestingly, the majority of an animal's dietary intake of protein/amino acids is through intake of plant-based proteins. In contrast to animal-based proteins, which have a well balanced amino acid composition essential for growth and development, plant-based proteins are nutritionally unbalanced and deficient in some essential amino acids. Therefore, plant-based protein diets require higher crude protein intake or supplementation with an animal protein source (derived from meat/fish and dairy processing) or specific amino acids (Beski *et al.* 2015). There are considerable advantages of reducing dietary crude protein with supplementation of free amino acids for sustainable livestock production, including saving on protein ingredients, reducing nitrogen excretion, feed costs and the risk of gut disorders, without impairing growth performance compared with traditional diets (Wang *et al.* 2018). Some amino acids with beneficial effects on skeletal muscle growth will now be discussed in detail.

Leucine

Muscle cells are highly sensitive to changes in amino acid availability, which plays a major role in the regulation of

protein synthesis and breakdown. Amino acid abundance results in enhanced activity of the mechanistic target of rapamycin complex 1 (mTORC1), which is one of the key regulators of protein turnover that drives protein synthesis and growth (Ham *et al.* 2014a). Of all amino acids, the branched-chain amino acid, leucine, is the most potent stimulator of mTORC1 and protein synthesis *in vitro* and *in vivo* (Ham *et al.* 2014a). As such, leucine has received considerable attention as a potential pharmaconutrient to enhance growth. Multiple studies have shown that administration of leucine or leucine-rich supplements acutely increases protein synthesis in mice and rats (Anthony *et al.* 2000), pigs (Murgas Torrazza *et al.* 2010), sheep (Schaefer *et al.* 1986) and healthy humans (Wall *et al.* 2013). Interestingly, long-term, placebo-controlled, isocaloric studies in adult humans have consistently shown no beneficial effect of leucine supplementation on skeletal muscle mass or function (Verhoeven *et al.* 2009). We have critically evaluated the therapeutic potential of leucine to attenuate the skeletal muscle wasting associated with ageing, cancer and immobilisation/bed rest (Ham *et al.* 2014a) and highlighted the impact of inflammation on amino acid sensing, mTOR activation and stimulation of protein synthesis (Ham *et al.* 2016). Leucine, as a standalone nutritional intervention, is not effective in preventing muscle wasting. In contrast, some studies in rapidly growing young pigs fed a protein-restricted diet have shown that feeding with leucine (or its metabolite β -hydroxymethylbutyrate) can enhance growth (Wan *et al.* 2016; Zheng *et al.* 2016). Using porcine myoblasts, *in vitro* studies have suggested that leucine induces a fast-to-slow fibre-type transition via AMP-activated protein kinase/SIRT1-mediated (Chen *et al.* 2019) or FOXO1-mediated (Zhang *et al.* 2019) signalling. In contrast, recent *in vivo* studies demonstrated that leucine feeding in piglets suppressed oxidative phosphorylation and fatty acid β -oxidation, with activation of glycolysis and slow-to-fast fibre-type transition (Fan *et al.* 2017). More detailed studies are needed to elucidate the effect of leucine feeding on muscle phenotype in animals used for meat production.

Arginine and citrulline

Citrulline is a non-proteinogenic amino acid (i.e. an amino acid not incorporated into protein with a unique inter-organ metabolism) and it plays a central role in the delivery of arginine to skeletal muscle (Moinard and Cynober 2007). Since citrulline is not metabolised in the gut, oral citrulline administration is more efficient in increasing plasma and muscle concentrations of arginine than is arginine feeding. The semi-essential amino acid arginine is critically involved in numerous physiological functions, including providing substrate to produce creatine, urea and nitric oxide (NO). NO is a key signalling molecule that stimulates release of growth factors such as insulin and growth hormone and plays a role in vasodilation (and, thus, nutrient delivery to the muscle), satellite cell activation, myoblast fusion and overload-induced skeletal-muscle hypertrophy (Ham *et al.* 2014b). Arginine availability clearly plays a role in the regulation of protein synthesis and skeletal muscle mass in both NO-dependent and NO-independent ways (Ham *et al.* 2014b).

Citrulline supplementation reduces muscle wasting in conditions of arginine deficiency. In rats, massive intestinal resection results in skeletal muscle arginine deficiency and muscle atrophy, while restoration of skeletal muscle arginine pools with citrulline improves muscle protein metabolism and attenuates muscle wasting (Osowska *et al.* 2004). We have demonstrated a direct role for arginine in the protection of skeletal muscle cells from cachectic stimuli in C2C12 myotubes *in vitro* (Ham *et al.* 2014a). Arginine reduced muscle wasting in a dose-dependent manner and modulated protein synthesis rates in a mTORC1-dependent manner (Ham *et al.* 2014a). We have also demonstrated a novel direct protective effect of L-citrulline on protein metabolism and skeletal muscle cell size that is not mediated by signalling through mTORC1 (Ham *et al.* 2015a). Interestingly, studies performed *in vivo* have demonstrated that citrulline treatment has no effect on therapeutically relevant outcome measures such as skeletal muscle mass and peak muscle force after 14 days of hind-limb immobilisation (Ham *et al.* 2015b). The effect of citrulline feeding on muscle growth in pigs, sheep and beef cattle has not been investigated in detail. In contrast, many studies have examined the effect of arginine supplementation in pigs and, although capable of improving some aspects of meat quality, arginine does not affect growth performance and carcass yield in growing–finishing pigs (Madeira *et al.* 2014, 2015, 2016; Hu *et al.* 2017).

Glycine and related compounds

Glycine is one of the non-essential amino acids often considered to be biologically neutral. However, studies have indicated that glycine exerts a range of physiological effects in numerous tissues and cell types *in vitro* and *in vivo* (Koopman *et al.* 2017). Glycine is a substrate for the production of glutathione, heme and creatine and, therefore, plays a role in overall antioxidant defence and metabolism. Glycine administration also modulates homeostasis by activating glycine-gated chloride channels in inflammatory cells, thereby effectively reducing $[Ca^{2+}]_i$, cytokine production, and whole-body (systemic) inflammation in several models (Zhong *et al.* 2003). Because inflammation plays a key role in the aetiology of many muscle-wasting conditions, we have tested the hypothesis that glycine supplementation represents a simple, safe and promising nutritional intervention for tackling skeletal-muscle wasting in many diseases and conditions. We have shown that glycine protects from wasting in mouse models of cancer cachexia (Ham *et al.* 2014c) and enhances the anabolic response to leucine during inflammatory conditions (Ham *et al.* 2016). Our observations are consistent with other studies, showing that glycine supplementation attenuated the inflammatory response to lipopolysaccharide in broiler chicks and enhanced average daily gains in bodyweight (Takahashi *et al.* 2008). A glycine-related compound that has received considerable interest is guanidinoacetic acid (GAA), a precursor of creatine. GAA is synthesised from arginine and glycine, and GAA supplementation improves growth performance (DeGroot *et al.* 2019), breast meat yield (Córdova-Noboa *et al.* 2018a) and reduces the severity of wooden breast myopathy in broilers (Córdova-Noboa *et al.* 2018b). An amino acid such as glycine or

its derivatives that modulate inflammation and metabolism will be valuable additions to nutritional interventions for livestock and aquaculture.

Choline

Choline is an essential water-soluble nutrient with multiple biological roles, including countering inflammation and oxidative stress, promoting neurotransmission and membrane composition and enhancing lipogenesis. One of the ways in which choline, and its derivative betaine, can modulate muscle homeostasis is by serving as a methionine precursor (via one-carbon metabolism) and in the regulation of methylation of DNA, histones and other proteins (Abbasi *et al.* 2017).

Choline deficiency is implicated in neurological disorders, fatty liver disease, atherosclerosis and muscle wasting (Zeisel *et al.* 1991). Choline supplementation can combat deficiencies and complications (Fischer *et al.* 2007). We recently tested the hypothesis that choline supplementation would be beneficial in *mdx* dystrophic mice, the most widely used murine model of DMD, which is the most severe of the muscular dystrophies (Alves *et al.* 2019). Choline administration attenuated the dystrophic pathology, with reductions in the expression of inflammatory markers, macrophages and collagen infiltration (Alves *et al.* 2019). Choline supplementation in broiler chickens during the grower and finisher period effectively improved the feed conversion ratio, carcass yield and moisture content of leg muscle (Jahanian and Ashnagar 2018). Similarly, betaine feeding improved growth performance in broiler chickens (Chen *et al.* 2018; Shakeri *et al.* 2018) and was effective for the resynthesis of methionine to sustain protein synthesis in pigs fed a methionine-restricted diet (McBreairty *et al.* 2016).

Pharmacological strategies to alter muscle attributes and phenotype

Because muscle mass relies on myoblast proliferation during prenatal (or prehatch) stages and fibre hypertrophy through protein synthesis and nuclei donation by satellite cells after birth (or hatch), pharmacological approaches to optimise cellular and molecular mechanisms of myogenesis and muscle development are important (Chen and Lee 2016). Technologies to control fat and muscle composition in livestock were reviewed by Sillence (2004) who provided a comprehensive evaluation of pharmacological strategies, including anabolic steroids, corticosteroid suppressors, β -adrenoceptor agonists (β -agonists), growth hormone (GH), insulin-like growth factor-I (IGF-I), adipokines, myostatin inhibition and selective androgen receptor modulators (Cesbron *et al.* 2017). Various combinations of these approaches can also be employed to promote and sustain muscle growth and alter lean meat-yield production, and marbling (Boles *et al.* 2009).

Hormonal growth-promoting agents ('promoters' or 'promotants') have been used for promoting muscle growth in farm animals (including cattle and pigs) and have been reported as beneficial for production efficiency, profit and reduced environmental effects, yet their effects on meat quality (particularly on measures of toughness) have yet to be resolved (Lean *et al.* 2018). The purpose of hormonal growth promotants, as described (somewhat cheekily) by Stephany

(2010), is 'to obtain more edible muscle meat for less money' but essentially to shift nutrient use towards carcass lean tissue deposition at the expense of adipose tissue (Johnson and Chung 2007). However, different countries around the world have enacted total bans or restricted the use of specific growth-promoting/growth-partitioning drugs because of their potential toxicity and carcinogenic properties (Leporati *et al.* 2014). Other countries still allow their use in animal production, reflecting changes in consumer preferences and international politics (Higgins 2004; Stephany 2010; Bonny *et al.* 2018; Farmer and Farrell 2018).

Testosterone (anabolic steroids) and growth hormone (GH)

The ability of the sex steroids testosterone, oestrogen and progesterone and their synthetic derivatives (nandrolone, trenbolone, melenogestrol and hexoestrol) to increase lean tissue growth in ruminants is undisputed (Sillence 2004; Dayton and White 2014). Although their use remains controversial, anabolic implants (containing estrogenic and trenbolone acetate combinations) are used routinely in some countries during the finishing phase of beef production to improve animal performance and feed efficiency (Reinhardt 2007; Duckett and Pratt 2014), despite implants having potentially adverse effects on carcass quality and eating quality, depending on dose and frequency (Garmyn and Miller 2014). Similarly, GH and recombinant GH can change carcass composition, especially in pigs, but only to a mild extent in cattle (Sillence 2004). Steroid-based growth promoters generally elevate local and circulating IGF-I concentrations through activation of steroid receptors and downstream signalling pathways, which influence proliferation and myogenic differentiation of muscle stem cells (satellite cells), increasing protein synthesis, and reducing protein degradation, with net protein accretion and muscle hypertrophy (Du 2014). Other studies have argued that GH stimulates muscle growth in cattle, in part, by stimulating protein synthesis in muscle through a GH receptor-mediated, IGF-I-independent mechanism, with liver-derived circulating IGF-I being the major mechanism mediating the growth-stimulatory effect of GH on muscle in cattle and other domestic animals (Jiang and Ge 2014).

β -adrenoceptor agonists

Although traditionally used for treating bronchospasm in animals and humans, it became apparent that stimulation of the β -adrenergic system with β -adrenoceptor agonists (particularly β_2 -agonists) had the ability to increase skeletal-muscle mass and decrease body fat. These so-called 'repartitioning effects' proved desirable for the livestock industry with the intention of improving feed efficiency and meat quality (Lynch and Ryall 2008). Although protein turnover rates can be augmented by β -agonists in humans (Hostrup *et al.* 2018), their muscle anabolic effects appear to be much less pronounced than those observed in livestock. Studies on cattle, sheep and pigs have shown that the tissue responsiveness to β -agonists varies from species to species, and even among different tissues within a species, primarily because of differences in the density of β -receptor subtypes (Lynch and

Ryall 2008). Many studies have examined the use of β -agonists in livestock, especially with respect to their potential to improve meat quantity and, to a lesser extent, quality, because they can increase toughness in beef loin (Sillence 2004; Dunshea *et al.* 2005). The anabolic effects of β -agonists attenuate as β -adrenoceptors in skeletal muscle downregulate, and, in some cases, sudden withdrawal (of some β -agonists) can result in a marked catabolic response (Sillence 2004). These factors influence how β -agonists might be used commercially to maximise muscle growth but also limit tissue residues of these compounds that may produce off-target (adrenaline-like) effects in consumers.

Two β -adrenergic agonists are approved for use in cattle fed in confinement for slaughter in the United States, namely, zilpaterol hydrochloride and ractopamine hydrochloride, with the purpose of increasing the rate of gain, improving feed efficiency and increasing carcass leanness (Delmore *et al.* 2010; Brown *et al.* 2014; Martin *et al.* 2014). Maximum residue limits for ractopamine determined by the Joint FAO/WHO Expert Committee on Food Additives (JECFA) were adopted by the Codex Alimentarius Commission (Codex), although no such limits have been determined for zilpaterol (Centner *et al.* 2014). Many countries disagree with the Codex standards and maintain a policy of restricting or banning meat products containing β -agonists (Centner *et al.* 2014), whereas other countries have fewer concerns. Methods are being developed to manage the safety of imported meat products from countries where zilpaterol use is still permitted, to prevent β -agonist poisoning due to secondary contamination (Sung *et al.* 2015). The consumption of contaminated meat products can lead to potentially serious side effects, including palpitations, peripheral vasodilatation, headache and cardiovascular complications, as well as tremor and muscle cramps (Lynch 2002).

On the basis of their growth-promoting effects on skeletal muscle, we have examined the therapeutic potential of several β -agonists (fenoterol, clenbuterol, formoterol) for muscle-wasting conditions, in animal (mouse, rat) models of muscular dystrophies and sarcopenia (Beitzel *et al.* 2007; Lynch and Ryall 2008; Ryall and Lynch 2008; Koopman *et al.* 2010). From a 'muscle paradox' perspective and attempting to elicit maximal muscle hypertrophy, we have found that these β -agonists typically cause a (dose-dependent) 10–20% increase in muscle mass within 2–4 weeks, after which the muscle-mass response reaches a plateau due to receptor downregulation and desensitisation. Because the heart also contains β -adrenoceptors (mainly β_1 -adrenoceptors but also some β_2 -adrenoceptors), β -agonist administration even with highly selective β_2 -agonists can trigger off-target complications such as cardiac hypertrophy (Ryall and Lynch 2008). From a therapeutic perspective, obviating the deleterious cardiovascular side effects of β -agonists remains an important challenge (Ryall and Lynch 2008).

Therefore, we investigated whether β -adrenoceptor-mediated signalling could be modulated in skeletal muscle via gene delivery to the target tissue, thus avoiding risks associated with β -agonists. In mice, intramuscular administration of a recombinant adeno-associated virus-based vector expressing the β_2 -adrenoceptor increased muscle mass by more than 20% within 4 weeks, a hypertrophic response comparable to that of

administration of formoterol for 4 weeks. Recombinant adeno-associated virus-based vectors are emerging tools for therapeutic gene delivery because of their capacity for efficacious and targeted delivery of transgenes to mammalian skeletal muscles (Hagg *et al.* 2016). The study showed that gene therapy-based interventions targeting the β_2 -adrenoceptor pathway could promote skeletal muscle hypertrophy independent of ligand administration, highlighting how these methods could be utilised for altering muscle mass, being relevant to treating muscle-wasting conditions, but also for livestock production.

Myostatin and follistatin

As described in the introductory sections of this review (see *Signalling pathways regulating muscle adaptation and plasticity*), β -agonist administration can increase skeletal-muscle mass in mammals, but the magnitude of hypertrophy is much less than what can be elicited with manipulation of the TGF- β superfamily signalling pathway, such as myostatin inhibition or increasing follistatin expression. Myostatin (GDF-8), a member of the TGF- β superfamily, is a negative regulator of myogenesis and suppresses myoblast proliferation and myogenic differentiation. Several animals, including cattle, sheep, dogs and humans, display the 'double-muscled' phenotype due to mutations in the myostatin gene and understanding of different null alleles and polymorphisms in the myostatin gene could be applied to improving meat production in livestock animals (Aiello *et al.* 2018). Myostatin positively regulates slow but negatively regulates fast myosin, such that in transgenic myostatin null mice, there is a shift towards faster isoforms (Wang *et al.* 2012). Even heterozygous myostatin-knockout pigs exhibit a disproportionate increase in muscle mass and more fast glycolytic muscle fibres than do wild type pigs (Xing *et al.* 2017).

Chen and Lee (2016) reviewed inhibitors of myostatin as methods of enhancing muscle growth and development for animal production, indicating that there are currently no commercial myostatin inhibitors for agriculture or biomedical purposes because safe and effective options are yet to be identified. They suggested that further investigation of myostatin inhibitors and administration strategies may revolutionise animal production and the medical field. For example, because myostatin exerts its actions on skeletal muscle via interaction with ActRIIB, inhibition of this receptor is an attractive therapeutic avenue for attenuating muscle wasting (Swiderski and Lynch 2015) but also for animal production. Blocking myostatin signalling through genetic and pharmacological approaches induces skeletal-muscle hypertrophy, whereas overexpression or systemic administration causes muscle atrophy (Lee *et al.* 2012). Myostatin signalling can be disrupted by neutralising antibodies to myostatin (Whittemore *et al.* 2003; Murphy *et al.* 2010), a modified myostatin propeptide to block myostatin (Bogdanovich *et al.* 2005), and a soluble ActRIIB receptor Fc fusion protein (Lee *et al.* 2005; Tsuchida 2008; Zhou *et al.* 2010; Attie *et al.* 2013). With respect to therapeutic applications, it should be noted that a randomised, double-blind, placebo-controlled, ascending-dose trial of the fusion

protein myostatin inhibitor, ACE-031, in DMD patients, although not associated with serious or severe adverse events, was stopped after the second dosing regimen due to potential safety concerns of epistaxis (nosebleeds) and telangiectasias ('spider veins'; Campbell *et al.* 2017).

Of most relevance to interrogating the 'muscle paradox' perspective, we undertook a series of investigations in mice on the effect of a myostatin-inhibiting antibody (PF-354) on skeletal muscle wasting, in settings of sarcopenia, unloading, muscular dystrophy and cancer cachexia (Murphy *et al.* 2011). Myostatin inhibition not only attenuated muscle atrophy in these wasting settings, but in otherwise healthy mice, it increased muscle fibre cross-sectional area by 12% and enhanced maximum force (function) of mouse tibialis anterior muscles by 35% (Murphy *et al.* 2010). Compared with transgenic myostatin null mice that exhibit a shift in muscle phenotype to having a larger proportion of fast Type II (glycolytic) fibres and a smaller proportion of slow Type I (oxidative) fibres than do wild-type mice (Girgenrath *et al.* 2005), we found that myostatin antibody (PF-354) treatment increased the proportion of type IIa (fast oxidative) fibres by 114% and enhanced the activity of oxidative enzymes (e.g. succinate dehydrogenase) by 39% (Murphy *et al.* 2010). Therefore, the effects of myostatin inhibition vary depending on the mode of intervention, indicating that it is possible (at least through an inhibitory antibody) to produce larger, more oxidative skeletal muscles; these findings were not predicted on the basis of the muscle paradox. Producing larger, more oxidative muscle fibres is favourable for animal production, ultimately producing greater yields and potentially superior flesh qualities.

Follistatin is a potent myostatin antagonist that acts via a pathway independent of the myostatin signalling cascade by inhibiting binding of myostatin to the ActRIIB. Therefore, administration of follistatin can enhance skeletal muscle mass with fewer off-target effects compared with administration of other myostatin inhibitors trialled previously (Swiderski and Lynch 2015; Hardee and Lynch 2019). Mice genetically engineered to overexpress follistatin, specifically in skeletal muscle, had at least twice the amount of muscle mass of control mice (Chang *et al.* 2017) and viral vector-mediated expression of follistatin in mouse skeletal muscles produced similar (or even greater) increases in muscle mass (Winbanks *et al.* 2012; Sepulveda *et al.* 2015; Davey *et al.* 2016). This makes follistatin attractive for studying the relationship between muscle growth and muscle phenotype, especially for exploring the limits of muscle fibre size.

Less is known about the role of follistatin in skeletal muscle development of livestock, but in pigs, muscle-specific follistatin overexpression enhanced skeletal muscle growth, highlighting its potential for increasing muscle mass in pigs and other livestock species (Chang *et al.* 2017). Transgenic rainbow trout overexpressing follistatin exhibited increased total muscle surface area with epaxial and hypaxial muscling similar to that observed in double-muscled cattle and myostatin null mice, being attributed to inhibition of myostatin and possibly other growth factors (Medeiros *et al.* 2009). The hypaxial muscling generated a phenotype with well developed abdominal and intercostal muscles (as in athletic humans) and was dubbed 'six pack'! (Medeiros *et al.* 2009).

With respect to understanding the muscle paradox and limitations on muscle fibre size, the increased musculing in the transgenic rainbow trout was attributed to hyperplasia, with more fibres per unit area and increases in the percentage of smaller fibres and the number of total fibres (Medeiros *et al.* 2009).

In addition to systemic or intramuscular adeno-associated virus follistatin delivery, nanoparticle-mediated delivery of follistatin mRNA to the liver after subcutaneous administration (Schumann *et al.* 2018) has emerged as an effective way to increase muscle mass, with potential relevance to animal production. After subcutaneous injection of mRNA-loaded nanoparticles, the mRNA accumulates and internalises in the liver, where the hepatic cellular machinery produces follistatin. Serum concentrations of follistatin remained elevated for 72 h after injection and reduced concentrations of activin A and myostatin, with repeated injections over 8 weeks being required to increase lean muscle mass by 10% compared with controls (Schumann *et al.* 2018). The nanoparticle delivery of follistatin, while not as efficacious for increasing muscle mass as a single adeno-associated virus injection, provides a way to transiently manipulate follistatin concentration that may prove desirable for animal-production applications.

Conclusions

The muscle paradox suggests fibre size and oxidative capacity are mutually exclusive, such that muscle fibres can hypertrophy at the expense of their endurance capacity. While skeletal muscle adaptations to perturbing stimuli generally obey this relationship, there are some situations (including genetic manipulation and pharmacological interventions) where this limitation can be overcome to produce larger, more oxidative muscle fibres.

On the basis of the evidence provided herein, extremes of muscle hypertrophy can be achieved, especially through manipulation of TGF- β signalling, including strategies that decrease myostatin and increase follistatin. Genetic selection of myostatin increases muscle fibre size but shifts muscle phenotype to being more glycolytic, which are attributes associated with tougher flesh and less desirable meat quality. Antibody-directed suppression of myostatin has, in some mammals (such as mice), caused muscle fibre hypertrophy and a concomitant increase in the overall muscle oxidative capacity, attributes that would not be predicted based on the muscle paradox. Viral vector methods and nanoparticle-mediated delivery of mRNA are powerful tools for manipulating biochemical signalling to direct muscle growth and phenotype. Combinatorial approaches may have potentially greater efficacy in animal production for selectively altering muscle attributes, ultimately to produce larger, more oxidative muscles with more desirable flesh qualities. Such strategies are theoretically applicable to farm animals from chickens to free range ruminants, recognising, of course, that safety concerns would need to be interrogated rigorously for the health and safety of both the animals being farmed and the consumers eating the meat products. This is especially relevant to some societal views and restrictions on the use of drugs, hormones or engineering methods to alter animal growth trajectories and flesh quality.

The capacity to overcome the paradox to enhance specific muscle attributes has important implications for agriculture and aquaculture; for ageing, occupational/work physiology, and sports performance; for the development of 'laboratory meat' or 'clean meat' and other synthetic foods; and for the engineering of bioartificial muscles and tissues with attributes that confer functionality and biological purpose.

Conflicts of interest

The authors declare no conflicts of interest.

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