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REVIEW

Prevention of muscle wasting and osteoporosis: the value of examining novel animal models

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ABSTRACT

Bone mass and skeletal muscle mass are controlled by factors such as genetics, diet and nutrition, growth factors and mechanical stimuli. Whereas increased mechanical loading of the musculoskeletal system stimulates an increase in the mass and strength of skeletal muscle and bone, reduced mechanical loading and disuse rapidly promote a decrease in musculoskeletal mass, strength and ultimately performance (i.e. muscle atrophy and osteoporosis). In stark contrast to artificially immobilised laboratory mammals, animals that experience natural, prolonged bouts of disuse and reduced mechanical loading, such as hibernating mammals and aestivating frogs, consistently exhibit limited or no change in musculoskeletal performance. What factors modulate skeletal muscle and bone mass, and what physiological and molecular mechanisms protect against losses of muscle and bone during dormancy and following arousal? Understanding the events that occur in different organisms that undergo natural periods of prolonged disuse and suffer negligible musculoskeletal deterioration could not only reveal novel regulatory factors but also might lead to new therapeutic options. Here, we review recent work from a diverse array of species that has revealed novel information regarding physiological and molecular mechanisms that dormant animals may use to conserve musculoskeletal mass despite prolonged inactivity. By highlighting some of the differences and similarities in musculoskeletal biology between vertebrates that experience disparate modes of dormancy, it is hoped that this Review will stimulate new insights and ideas for future studies regarding the regulation of atrophy and osteoporosis in both natural and clinical models of muscle and bone disuse.

KEY WORDS: Aestivation, Apoptosis, Hibernation, Immobilisation, Protein synthesis, Sclerostin

Introduction

The vertebrate musculoskeletal system executes an array of important functions, including facilitating movement and breathing, providing a structure to support an upright stance and protecting internal organs. Bone often serves as a reservoir for calcium and, in some animals, is also the predominant site of haematopoiesis (Renkema et al., 2008). Skeletal muscle accounts for >80% of glycogen storage and is crucial for glucose homeostasis and the regulation of metabolism (Jensen et al., 2011).

Skeletal muscle and bone mass are influenced by various factors, including diet and nutrition, genetics, hormones, growth factors and mechanical stimuli (Goodman et al., 2015). The mass of bone and skeletal muscle is maintained in proportion to the mechanical loading experienced by the musculoskeletal system. For example, resistance training leads to an increase in muscle size and bone mineral density, which is associated with improved musculoskeletal fitness (English et al., 2014; Ryan et al., 2004). In contrast, conditions of low mechanical load and disuse, such as cast immobilisation, prolonged bed rest and hindlimb unloading, promote significant losses of both skeletal muscle and bone mineral mass and strength (Bloomfield, 1997; Bodine, 2013a; Veitch et al., 2006). The deleterious effects of prolonged musculoskeletal disuse (i.e. muscle atrophy and osteopenia or osteoporosis; see Glossary) appear to be common to the majority of vertebrates studied, but the degree of loss is variable and dependent on factors such as age, the extent of disuse, and muscle fibre or bone type composition (Hudson and Franklin, 2002b; McGee-Lawrence et al., 2008).

It is well known that numerous animals naturally enter prolonged periods of dormancy (e.g. hibernation and aestivation; see Glossary) to survive unfavourable environmental conditions; these periods usually involve prolonged inactivity and fasting. Remarkably, hibernating mammals and aestivating frogs have been shown to exhibit negligible or no loss of musculoskeletal mass and strength, despite the fact that they experience natural periods of extended inactivity and starvation that can, in extreme cases, last for years (Cotton, 2016; Hudson and Franklin, 2002b; James, 2010; McGee-Lawrence et al., 2008). The physiological adaptations of hibernating and aestivating animals may provide insight into why inactivity-induced muscle and bone loss occurs relatively rapidly in other vertebrate species, and might suggest novel potential treatments for these conditions in humans. Treatment options for muscle disuse atrophy and osteoporosis are currently limited, with rehabilitative strength training proving to be the most effective method to restore musculoskeletal mass; however, long periods of rehabilitation are necessary to restore muscle, bone and locomotor performance following artificial immobilisation in non-hibernating mammals. This Review will firstly provide an overview of musculoskeletal disuse in vertebrates by describing the defining features of disuse-induced muscle atrophy and osteoporosis, summarising the recent developments in understanding the potential underpinning mechanisms. We then focus on the exciting and novel physiological and cellular strategies used by dormant animals to preserve musculoskeletal integrity. We conclude by discussing potential future directions: it is clear that additional whole-animal and systems biology approaches are required before we fully understand the aetiology of the disuse or dormancy-associated musculoskeletal phenotype.

Disuse-induced bone loss

Osteoporosis is a disorder of the skeleton characterised by decreased bone mass and deterioration of the microarchitecture of bone tissue, resulting in increased bone fragility and probability of fracture. Osteopenia is less severe and refers to bone density that is below

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Glossary

Aestivation
A summer or dry season form of dormancy. Desiccating conditions that limit water and food availability typically trigger aestivation, often but not always in conjunction with hot summer temperatures.

Dormancy
A state of transiently reduced metabolic activity.

Hibernation
A physiological state usually characterised by metabolic depression and a decreased body temperature set point to conserve energy during periods of low food availability and unfavourable environmental conditions. Some temperate-zone hibernators, such as ground squirrels, must intermittently arouse back to euthermia (i.e. proper body temperature), a process referred to as interbout arousal (IBA).

Hypercalciuria
The excretion of unusually high levels of calcium in the urine.

Osteoblast
Cell from which bone develops; a bone-forming cell.

Osteoclast
Large multinucleated cell associated with the absorption and elimination of bone.

Osteocyte
A mature osteoblast that has become embedded in the bone matrix.

Osteopenia
A reduction in the calcification or density of bone to a level that is below normal peak density, but less severe than that resulting from osteoporosis.

Osteoporosis
A decrease in the quantity of bone or atrophy of skeletal tissue. Osteoporosis is a common age-related disorder characterised by reduced bone mass and loss of normal skeletal microarchitecture and strength, leading to increased risk of bone fracture.

Sarcopenia
Loss of skeletal muscle mass (atrophy) associated with ageing.

Satellite cell
Mononuclear, undifferentiated cell located in skeletal muscle fibre and involved in skeletal muscle growth, repair and regeneration.

Tenotomy
Surgical cutting or division of a tendon.

normal peak density but not low enough to be classified as osteoporosis. Loss of bone due to chronic inactivity or decreased mechanical demand (e.g. due to plaster cast immobilisation, bed rest or hindlimb suspension) has been demonstrated in multiple vertebrate species, including humans, monkeys, mice, rats, turkeys, dogs and sheep (Gross and Rubin, 1995; Li et al., 2005a; Lloyd et al., 2012; Rubin et al., 1988; Turner et al., 2006; Young et al., 1983) (Table 1). Cast immobilisation for as little as 10 days has been shown to result in osteopenia in rodents, as evidenced by decreased dry bone mass, bone mineral density and metaphyseal bone volume (Delling et al., 1970; Hott et al., 2003; Rantakokko et al., 1999; Weinreb et al., 1989). Similarly, in turkeys, 8 weeks of bone disuse resulted in a 12% loss in total bone area, which was uniform along the diaphyseal shaft (Gross and Rubin, 1995). Weight-bearing bones are particularly sensitive to the absence of mechanical load, with the proximal femur and tibia exhibiting ∼5% and 23% reductions in bone mineral density following 3 and 6 months of disuse, respectively (Watanabe et al., 2004; Young et al., 1983). Consequently, the rigidity of bone, which is correlated with bone mineral density, also declines during experimental disuse.

Balancing formation and resorption: osteoblasts and osteoclasts

The mass of bone is regulated by the balance between the activity of osteoblasts and osteoclasts (see Glossary), which form and resorb bone, respectively. Skeletal unloading promoted by both cast immobilisation and hindlimb suspension results in imbalanced rates of bone turnover, leading to bone loss (Tuukkanen et al., 1991; Uhthoff and Jaworski, 1978; Weinreb et al., 1989; Young et al., 1986) (Table 1) and subsequent transient hypercalciuria (see Glossary; Watanabe et al., 2004). Histomorphometric and biochemical studies have demonstrated that immobilisation-induced bone loss occurs predominantly because of an increase in bone resorption associated with a decrease in bone formation (Bagi and Miller, 1994; Rantakokko et al., 1999; Weinreb et al., 1989). More recently, bone loss following 90 days of bed rest in humans was shown to result from a dramatic elevation in bone resorption, associated with only a mild increase in bone formation (Watanabe et al., 2004). In hindlimb unloading experiments, where rodents are suspended by their tails, bone formation is generally inhibited, whereas bone resorption is enhanced or remains unchanged (Apseloff et al., 1993; Bikle et al., 1994; Globus et al., 1986; Moriishi et al., 2012; Simske et al., 1994; Vico et al., 1991; Wronski and Morey, 1982). Interestingly, the susceptibility to bone loss due to unloading is largely dependent on genetic background in mice, with the common laboratory inbred mouse strain (C57BL/6) consistently exhibiting markedly reduced bone formation and enhanced bone resorption (Judex et al., 2004).

Osteocytes and sclerostin

The adaptive response of bone to variations in loading is controlled by the capacity of resident bone cells to sense and translate mechanical energy into a cascade of structural and biochemical alterations within the cells, a process known as mechanotransduction. Osteocytes (see Glossary), the most prevalent cells in mature bone, are the primary cell type responsible for sensing mechanical load and translating it into cell signalling and downstream bone remodelling (Santos et al., 2009). Indeed, genetically modified mice lacking osteocytes develop osteoporosis as a result of defective mechanotransduction (Tatsumi et al., 2007). The canonical Wnt/β-catenin signalling pathway plays a significant role in modulating bone

Table 1. Animal models and experimental methods used to generate and study disuse-induced bone osteoporosis or osteopenia and associated unbalanced bone remodelling

Disuse model	Animal model	Bone type	References
Hindlimb suspension	Mouse (*Mus musculus*)	Tibia, femur	Lloyd et al., 2012
	Rat (*Rattus norvegicus*)		Turner et al., 2006
Limb immobilisation (i.e. splint or plaster casting)	Rat (*Rattus norvegicus*)	Tibia, femur	Hott et al., 2003
	Dog (*Canis familiaris*)	Metacarpal	Li et al., 2005a
Bed rest/prolonged semi-recumbent position	Human (*Homo sapiens*)	Head, forearm, lumbar spine, femur	Watanabe et al., 2004
	Monkey (*Macaca mulatta*)	Tibia	Young et al., 1983
Tenectomy	Sheep (*Ovis aries*)	Calcaneus	Rubin et al., 1988
Osteotomy	Turkey (*Meleagris gallopavo*)	Radius	Gross and Rubin, 1995
Sciatic neurectomy	Rat (*Rattus norvegicus*)	Tibia	Brighton et al., 1988

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mass through regulating processes including osteoblast differentiation and function (MacDonald et al., 2009). Additionally, the glycoprotein sclerostin (encoded by the *SOST* gene), which is expressed in osteocytes, has been identified as a negative regulator of bone growth that acts by inhibiting Wnt signalling and bone formation (Li et al., 2005b) (Fig. 1). Sclerostin binds to low-density lipoprotein receptor-related proteins 5 and 6 (LRP5/6) and, as a result, inhibits osteoblast differentiation, activity and survival (Baron and Rawadi, 2007). Several studies support the concept that osteocytes possess mechanosensory functions, with sclerostin acting as a primary signalling protein. Mice lacking a functional *SOST* gene exhibit increased bone formation and strength (Li et al., 2008), and loading of forearm bone is correlated with decreased *SOST* mRNA and protein levels (Robling et al., 2008). These data were complemented by experiments demonstrating that *SOST* gene expression levels increase during tail suspension experiments (Robling et al., 2008; Tatsumi et al., 2007). In addition, application of an antibody against sclerostin was shown to inhibit disuse-induced bone deterioration in mice, whereas serum levels of sclerostin were markedly elevated during bed rest (for 90 days) in healthy human males (Spatz et al., 2013, 2012). Such observations in both humans and laboratory animals have resulted in the investigation of sclerostin inhibition as a possible means of treating osteoporosis.

Extra-skeletal regulators of bone mass

Cell pathways involving molecules produced outside of the skeleton are also involved in controlling bone mass and turnover. Bone remodelling is regulated by rich innervation of the skeleton, providing a source of various hormones, growth factors and neurotransmitters that modulate various functions of bone. For recent reviews on this topic, see Niedzwiedzki and Filipowska (2015) and Quiros-Gonzalez and Yadav (2014). Important extra-skeletal compounds that are relevant to this Review and which can regulate bone remodelling include serotonin and the hormone leptin. Although leptin is usually associated with controlling appetite and body mass, studies have shown that it also negatively regulates bone mass by inhibiting serotonin production and release in a negative-feedback loop (Yadav et al., 2009).

Leptin may prevent excessive bone resorption by acting through a neuropeptide known as cocaine- and amphetamine-regulated transcript (CART). Elefteriou et al. (2005) found that CART reduces the expression of receptor activator of nuclear factor kappa-B ligand (RANKL), a key factor involved in osteoclast differentiation and activation (Fig. 1). Furthermore, overexpression of peripheral CART was shown to inhibit the development of osteoclasts and rescue the low bone mass phenotype characteristic of CART-knockout mice (Singh et al., 2008). Although we have only briefly touched on the involvement of neuronal circuits in regulating bone, there are multiple new molecular players implicated in the control of bone mass that represent an active area of research, including brain-derived neurotrophic factor (BDNF) and interleukin-1 (IL-1) (Quiros-Gonzalez and Yadav, 2014). For example, BDNF and its receptors are highly expressed in osteoblasts, and central BDNF deletion was associated with increased whole-bone mineral density and bone mineral content in mice (Quiros-Gonzalez and Yadav, 2014). In contrast, administration of IL-1 to normal mice enhances bone resorption and causes hypercalcaemia, while mice lacking IL-1 receptors exhibit a low bone mass phenotype (Sato et al., 1989).To briefly summarise, immobilisation and unloading promote bone loss secondary to an increase in resorption and decreased formation in

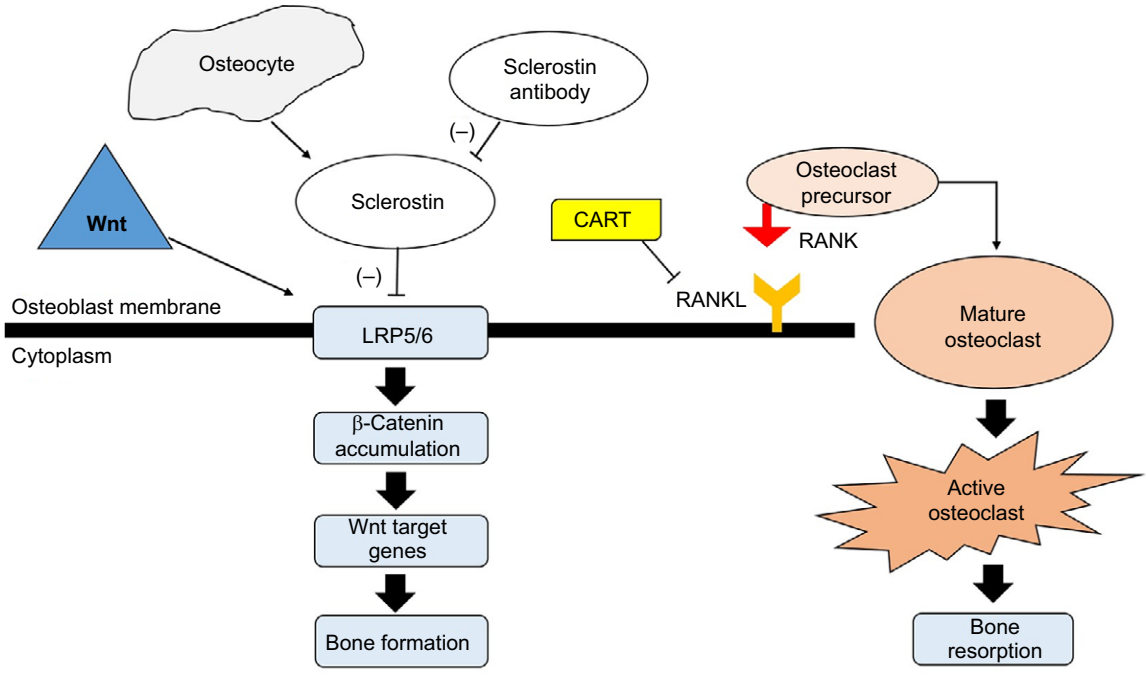

Fig. 1. Functions of canonical Wnt/β-catenin signalling, sclerostin and receptor activator of nuclear factor kappa-B ligand (RANKL) in bone remodelling. The balance between bone formation and bone resorption is primarily modulated by Wnt/β-catenin signalling (bone formation), sclerostin (negative regulator of bone formation) and RANKL (osteoclast activation). Binding of Wnt proteins to low-density lipoprotein receptor-related proteins 5/6 (LRP5/6) on the cell membrane of osteoblasts results in stabilisation of β-catenin and regulation of target genes that act to stimulate increased bone formation. Sclerostin, which is secreted by osteocytes, may bind to LRP5/6 and inhibit Wnt signalling, thus preventing bone formation. RANKL present on the osteoblast membrane binds RANK present on osteoclast precursors, and this promotes osteoclast maturation and resorption of bone. A neuropeptide called cocaine- and amphetamine-regulated transcript (CART) can decrease the expression of RANKL.

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weight-bearing bones. Moreover, skeletal muscle atrophy often occurs in parallel to bone loss and activity; muscle mass and strength influence bone mass (Swift et al., 2010). However, because the mechanisms that regulate mechanotransduction in bone and muscle are complex, it is not entirely clear which specific mechanisms operate in synergy during musculoskeletal disuse (Goodman et al., 2015).

Disuse-induced muscle atrophy

Skeletal muscle is a highly plastic tissue; that is, it can change in response to sustained stimuli. Like bone, the loss or 'wasting' of skeletal muscle occurs during long periods of disuse (e.g. lack of physical exercise, cast immobilisation, unloading or extended bed rest) and has been reported in humans, mice, rats, guinea pigs, cats and dogs (Bloomfield, 1997; Bodine, 2013a; Hudson and Franklin, 2002b) (Table 2). On a gross level, this manifests as a loss of whole-muscle mass and reduced cross-sectional area of muscle fibres, while at the subcellular level there is an overall loss of proteins, cytoplasm and mitochondria. In this section, we will introduce some of the key mechanisms implicated in the progression of muscle disuse atrophy (Fig. 2).

Regulators of skeletal muscle mass

The mass of skeletal muscle is determined by the difference in the rates of protein degradation and protein synthesis. Muscle hypertrophy is the result of an increase in protein synthesis and/or a reduction in protein degradation, whereas muscle atrophy occurs through a reduction in protein synthesis and/or an increase in the rate of protein degradation. Several protein degradation pathways contribute to the proteolysis of muscle proteins, including the ubiquitin–proteasome system, Ca^{2+}-dependent proteases (calpains), the autophagy–lysosome system and caspases. Interactions among these pathways appear to be critical in promoting proteolysis during muscle disuse atrophy (Powers et al., 2011; Talbert et al., 2013), while reactive oxygen species (ROS) have been implicated as a primary trigger leading to proteolysis under disuse conditions (Powers et al., 2011; Talbert et al., 2013). Gene expression profiling of disused muscle has demonstrated both increases and decreases in the expression of numerous genes over time, indicating that a complex suite of biochemical changes occur simultaneously to induce muscle atrophy (Chen et al., 2007; Stevenson et al., 2003). It is beyond the scope of this Review to analyse all of the cellular pathways implicated in the progression of muscle disuse atrophy in depth, so readers are directed to several recent reviews on the topic (Bodine, 2013a; Brooks and Myburgh, 2014; Powers et al., 2011; Schiaffino et al., 2013). In the remainder of this section, we focus on summarising how protein synthesis is affected during muscle disuse atrophy, specifically discussing IGF1–Akt–mTOR signalling and myostatin, as well as the importance of myonuclear apoptosis.

The role of mTOR in muscle remodelling

The retention of muscle mass and myofibre hypertrophy are, to some extent, regulated by insulin-like growth factor-1 (IGF1) signalling, as IGF1 can stimulate muscle growth via phosphatidylinositol 3-kinase–Akt signalling (PI3K/Akt) (Rommel et al., 2001). Active Akt has many significant downstream actions; for example, it modulates the phosphorylation of mTOR (mammalian target of rapamycin), a protein kinase that mediates various processes involved in cell proliferation and growth, including protein synthesis (Goodman, 2014). Extensive human and rodent studies have revealed the role of mTOR in the modulation of skeletal muscle mass and protein synthesis in response to different mechanical stimuli (Goodman, 2014). Direct activation of Akt is associated with skeletal muscle hypertrophy, and has also been shown to preserve muscle fibre size in skeletal muscles undergoing atrophy (Bodine et al., 2001). A decrease in the levels of native and phosphorylated Akt is evident during the atrophy accompanying hindlimb unloading, and theoretically this could promote reduced mTOR activity and a subsequent reduction in protein synthesis (Bodine et al., 2001). P70S6 kinase and the eukaryotic initiation factor 4E (eIF4E)-binding protein (4E-BP1) are two other important proteins that can be regulated through phosphorylation by mTOR. For example, P70S6 kinase is activated upon phosphorylation, promoting the formation of protein translation initiation complexes. Data indicate that P70S6 kinase and 4E-BP1 are involved in the decreased translation rate observed during disuse atrophy (Jackman and Kandarian, 2004). Curiously, a counterintuitive upregulation of mTOR has been reported during muscle inactivity induced by denervation, which might be due to increased amino acid influx from protein degradation pathways, such as the ubiquitin–proteasome system (Quy et al., 2013). Thus, although the precise role of mTOR-related signalling throughout different systems of muscle atrophy remains contentious, mTOR is still considered to be very important in modulating many cellular processes during muscle remodelling (Goodman, 2014).

Myostatin negatively regulates muscle growth

Another signalling pathway that controls skeletal muscle growth involves myostatin, a protein that acts as a negative regulator of muscle growth and size in adult mammals (Elliott et al., 2012). Blocking the myostatin pathway using various techniques has been shown to lead to muscle hyperplasia and hypertrophy (Zhu et al., 2000). Myostatin is suspected to play a role in muscle atrophy during disuse, with some studies demonstrating elevated abundance

Table 2. Animal models and experimental methods used to generate and study disuse-induced skeletal muscle atrophy and associated unbalanced protein turnover

Disuse model	Animal model	Bone type	References
Hindlimb suspension	Rat (*Rattus norvegicus*)	Plantar flexors	Swift et al., 2010
	Mouse (*Mus musculus*)	Soleus	Ferreira et al., 2008
Limb immobilisation (i.e. joint immobilisation or plaster casting)	Cat (*Felis catus*)	Tibialis posterior	Nordstrom et al., 1995
	Dog (*Canis familiaris*)	Rectus femoris, vastus lateralis, vastus medialis	Lieber et al., 1988
	Guinea pig (*Cavia porcellus*)	Soleus, gastrocnemius	Maier et al., 1976
	Human (*Homo sapiens*)	Gastrocnemius	Chen et al., 2007
Bed rest	Human (*Homo sapiens*)	Vastus lateralis	Brocca et al., 2012
Detraining	Human (*Homo sapiens*)	Quadriceps	Andersen et al., 2005
Denervation	Mouse (*Mus musculus*)	Extensor digitorum longus	Stockholm et al., 2001

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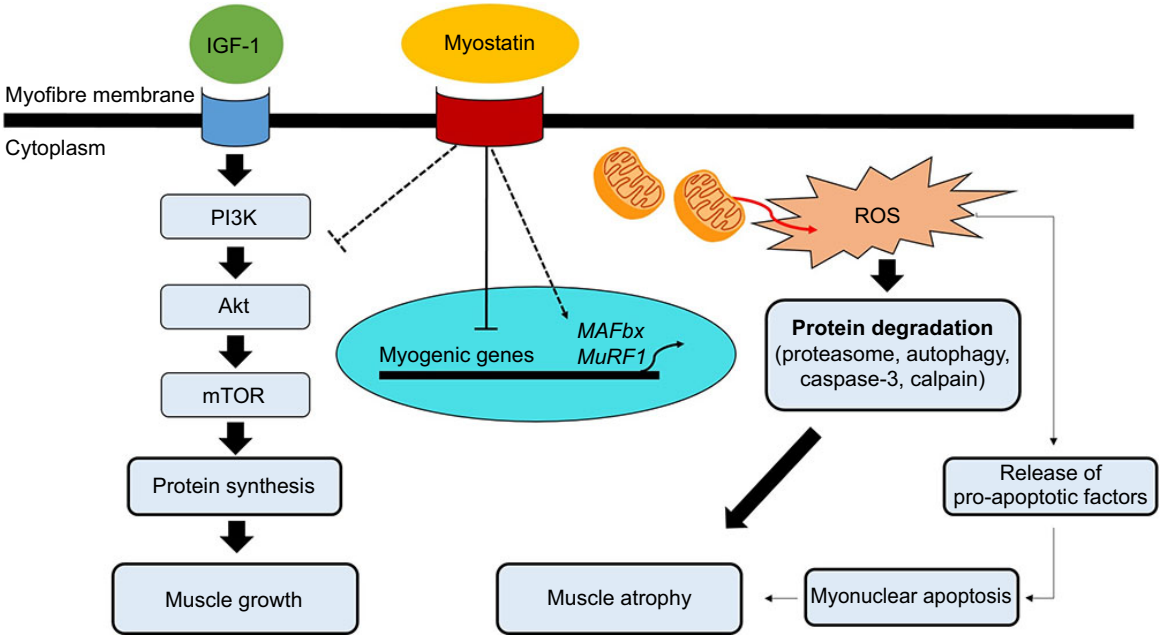

Fig. 2. Signalling pathways associated with muscle growth and muscle disuse atrophy. Insulin-like growth factor-1 (IGF-1) stimulates muscle growth via phosphatidylinositol 3-kinase–Akt signalling (PI3K/Akt). Active Akt has many significant downstream actions, including modulating the phosphorylation of mammalian target of rapamycin (mTOR), a protein kinase that regulates processes including cell proliferation, protein synthesis and muscle growth. Myostatin, a well-known negative regulator of muscle growth and size, is able to repress the PI3K/Akt/mTOR pathway. Binding of myostatin to its receptor also blocks the transcription of genes responsible for myogenesis, and can activate *MAFbx* (also known as atrogin-1) and *MuRF1*, two key proteolytic genes consistently upregulated in different muscle atrophy models. Skeletal muscle atrophy can be induced by mitochondrial production of reactive oxygen species (ROS), which stimulates protein degradation via a number of key pathways, including the ubiquitin–proteasome system, autophagy, calpains and caspase-3. Mitochondrial ROS production can also induce permeabilisation of the mitochondrial membrane, resulting in the release of pro-apoptotic factors, including cytochrome *c* and apoptosis-inducing factor. The release of cytochrome *c* can subsequently activate caspase-3, which may promote myonuclear apoptosis. Dashed lines and arrows represent the existence of intermediate steps, which have been omitted from the figure for simplicity.

of myostatin mRNA and protein in disused muscles (Reardon et al., 2001; Wehling et al., 2000). In addition, experimental inhibition of myostatin in mouse skeletal muscles unloaded for 14 days significantly decreases the extent of muscle atrophy (Murphy et al., 2011). Myostatin signalling can activate *MAFbx* (also known as atrogin-1) and *MuRF1*, two genes consistently upregulated in different muscle atrophy models (Elliott et al., 2012). In mammalian myoblasts, myostatin stimulates withdrawal from the cell cycle and promotes a quiescent state rather than differentiation or apoptosis (Brooks and Myburgh, 2014). This is achieved by inhibiting the activity of MyoD, which plays a fundamental role in regulating muscle differentiation (Elliott et al., 2012). Additionally, myostatin inhibits activation of the Akt/mTOR protein synthesis pathway (Trendelenburg et al., 2009); therefore, decreased myostatin gene expression may facilitate mTOR signalling by way of reducing this inhibition.

Apoptotic processes during muscle disuse atrophy

Like osteoclasts, myofibres are multinucleated cell types. This feature of skeletal muscle has led to the concept of the myonuclear domain theory, which describes the theoretical amount of cytoplasm within a myofibre that can be regulated by a single myonucleus (Fig. 3). Under conditions of muscle growth, hypertrophy or overload, there is an increase in myonuclear number as the muscle fibre increases in size (van der Meer et al., 2011). The responses of both myonuclear number and domain size with muscle atrophy are less well understood, although loss of myonuclei from existing fibres has been reported during skeletal muscle disuse (Allen et al.,

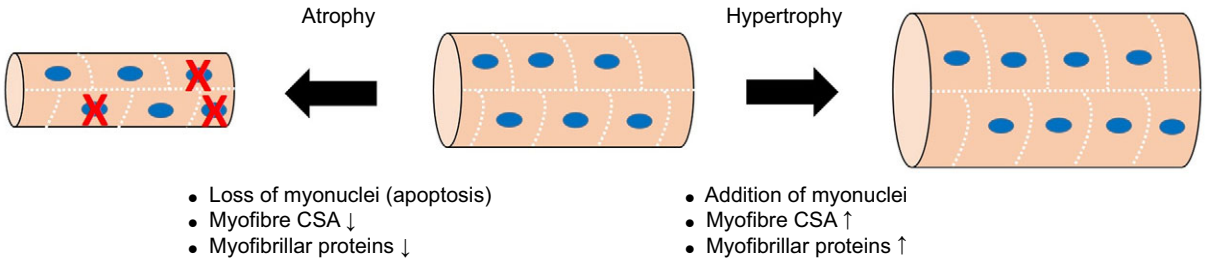

Fig. 3. Myonuclear gain and loss during skeletal muscle hypertrophy and atrophy. As myofibres grow in response to hypertrophic stimuli, myonuclei are recruited to support an increase in the volume of the cytosol. It has been suggested that, under atrophic conditions, nuclei are eliminated from the symplasm by myonuclear apoptosis (red crosses). During hypertrophy or disuse atrophy, alterations in the number of myonuclei coincide with changes in myofibrillar protein content and myofibre cross-sectional area (CSA). This results in a constant ratio of cytoplasmic area to nuclear number, i.e. constant size of the myonuclear domains (depicted in the figure by dashed white lines). Note that apoptosis as a mechanism for myonuclear loss during muscle disuse atrophy is controversial.

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1997; Gallegly et al., 2004; Smith et al., 2000). This may be due to apoptotic-like processes (Fig. 3). The defining features of apoptosis are consistent in most cell types (e.g. cell membrane blebbing, DNA fragmentation, phosphatidylserine externalisation), and cultured myocytes exhibit such hallmark morphological and biochemical characteristics in response to apoptotic stimuli (Adhihetty and Hood, 2003). As skeletal muscle is multinucleated, the likelihood of extensive myofibre death is relatively low, whereas individual myonuclear loss is probably a more common event within muscle tissue, ultimately leading to cellular remodelling.

Rats experiencing 2 weeks of hindlimb suspension exhibit an increase in the number of morphologically abnormal myonuclei and myonuclei showing double-stranded DNA fragmentation (as quantified using the TUNEL assay) (Allen et al., 1997) (Table 3). During similar prolonged periods of skeletal muscle disuse, rats also exhibit a reduction in myonuclear number and domain size, a decrease in the activity of satellite cells (see Glossary) in muscle fibres, and increases in several molecular markers of apoptosis, including elevated expression of Bax and Bcl-2 protein, increased mitochondrial cytochrome *c* content in the cytosol and nuclear colocalisation of EndoG (an apoptotic effector) in myonuclei (Dupont-Versteegden et al., 2006; Leeuwenburgh et al., 2005; Siu et al., 2005; Wang et al., 2006) (Table 3). Maximal caspase-3 and apoptosis-inducing factor expression was found in the soleus muscle of mice after 12 and 24 h of hindlimb suspension, respectively, which coincided with the highest levels of apoptotic DNA fragmentation (Ferreira et al., 2008). Further evidence that apoptosis is involved in the progression of muscle disuse atrophy arises from unilateral limb immobilisation studies in rats, mice and rabbits (Smith et al., 2000; Vazeille et al., 2008; Zhu et al., 2013), and a bird muscle loading/unloading model (Alway et al., 2003) (Table 3).

However, apoptosis as a mechanism for causing myonuclear loss during muscle atrophy is highly controversial, as recent studies using *in vivo* time-lapse imaging found that the number of myonuclei remained relatively constant during both tenotomy (see Glossary) and detraining (Gundersen, 2016). Whereas *in vivo* imaging allows direct examination of myonuclei, many studies have reported markers for apoptosis in homogenates derived from atrophying muscle and have interpreted this as apoptosis of myonuclei, but these findings could reflect apoptosis of nuclei from other cell types, such as stromal cells or satellite cells (Gundersen, 2016). Indeed, a recent study found that although the amount of Bax mRNA and the number of TUNEL-positive nuclei were significantly increased in immobilised human muscle, this was mainly localised in the interstitial space between myocytes, thus potentially being attributable to other cell types (Suetta et al., 2012). Nonetheless, a decrease in myonuclei has been observed during

Table 3. Morphological and molecular responses implicating apoptosis during muscle disuse atrophy in conventional experimental models

Disuse model	Species	Muscle	Summary	References
Hindlimb suspension (14 days)	Rat (*Rattus norvegicus*)	Soleus	TUNEL-positive staining in myonuclei ↑	Allen et al., 1997
		Soleus	Apoptotic DNA fragmentation ↑	Leeuwenburgh et al., 2005
		Gastrocnemius	Bax and Bcl-2 protein ↑ Mitochondrial cytochrome *c* release ↑	Siu et al., 2005
Hindlimb suspension (16 days)		Soleus	Satellite cell activity ↓ Myonuclear number ↓ Myonuclear domain size ↓	Wang et al., 2006
Hindlimb suspension (7 days)		Soleus	TUNEL-positive staining in myonuclei ↑ EndoG-positive staining in myonuclei ↑	Dupont-Versteegden et al., 2006
Hindlimb casting (8 days)		Gastrocnemius	TUNEL-positive staining in myonuclei ↑ Caspase-3 activity ↑ xIAP protein* ↓	Vazeille et al., 2008
Hindlimb suspension (48 h)	Mouse (*Mus musculus*)	Soleus	Caspase-3 activity ↑ Apoptosis-inducing factor expression ↑ Apoptotic DNA fragmentation ↑	Ferreira et al., 2008
Hindlimb casting (14 days)		Soleus	TUNEL-positive staining in myonuclei ↑	Zhu et al., 2013
		Gastrocnemius	Caspase-3 protein ↑	
Hindlimb casting (6 days)	Rabbit (*Oryctolagus cuniculus*)	Soleus	TUNEL-positive staining in myonuclei ↑ Presence of condensed chromatin Presence of irregularly shaped myonuclei	Smith et al., 2000
Wing unloading	Quail (*Coturnix coturnix*)	Patagialis	Presence of PARP‡-positive nuclei Caspase-3, -7, -8, -10§ activity ↑	Alway et al., 2003
Leg cast immobilisation (4 days)	Human (*Homo sapiens*)	Vastus lateralis	TUNEL-positive staining in myonuclei ↑¶ Bax mRNA ↑	Suetta et al., 2012

*xIAP (aka BIRC4) protein prevents apoptotic cell death; ‡poly(ADP-ribose) polymerase (PARP) can induce cell death by stimulating mitochondria to release apoptosis-inducing factor; §caspases-3 and -7 execute apoptosis whereas caspases-8 and -10 are involved in initiating apoptosis; ¶restricted to the interstitial space between myocytes.

other atrophy-inducing conditions, such as spinal cord isolation and transection, microgravity, sarcopenia (see Glossary) and neuromuscular pathologies (Brooks and Myburgh, 2014). Thus, elimination of myonuclei by nuclear apoptosis remains a rational concept to explain the regularity of myonuclear domain size regardless of atrophy (Brooks and Myburgh, 2014).

Dormant animals as model systems

There are a plethora of molecular mechanisms implicated in the events leading to musculoskeletal loss, and the deterioration of these tissues due to prolonged disuse appears to affect most vertebrates studied thus far. Not surprisingly, this includes a relatively limited variety of mammalian model systems (typically rodents and humans) which have been artificially immobilised in a laboratory or clinical setting. Some of the deleterious effects of musculoskeletal disuse may be reversed upon the return of normal weight bearing of the limbs, but this can depend on factors such as age, the duration and/or method of immobilisation and the type of bone or muscle (Jaworski and Uhthoff, 1986). Currently, there are limited therapeutic options for the treatment of osteoporosis and muscle disuse atrophy, because of an incomplete understanding of the cellular and molecular mechanisms involved in the induction and maintenance of these conditions. Understanding the physiology of animals that undergo natural periods of disuse but suffer negligible musculoskeletal deterioration could not only reveal novel regulatory factors but also lead to new therapeutic options.

Maintenance of musculoskeletal properties in hibernators and aestivators

It is well known that minimal loss of musculoskeletal mass and function occurs in hibernating mammals, despite the fact that they experience suppressed neural activity and natural periods of prolonged musculoskeletal disuse (Bodine, 2013b; Cotton, 2016; McGee-Lawrence et al., 2008). This applies to many species, such as bears, squirrels, woodchucks, bats, hamsters, prairie dogs and marmots (Cotton, 2016; Cotton and Harlow, 2010; Doherty et al., 2012; James et al., 2011; McGee-Lawrence et al., 2015, 2008; Utz et al., 2009; Wojda et al., 2012). However, some small hibernators, such as the 13-lined ground squirrel (*Ictidomys tridecemlineatus*) have been reported to exhibit microstructural bone loss (osteocytic osteolysis) during hibernation bouts, which has not been observed in larger hibernating animals (McGee-Lawrence et al., 2011).

Although hibernating mammals have been widely used as ‘natural analogues’ to which clinical disuse models such as bed rest or cast immobilisation may be compared, vertebrates that undergo aestivation also experience extended bone and muscle disuse during bouts of dormancy. This includes aestivating frogs, fish and reptiles. Several species of arid-zone frogs (e.g. *Cyclorana alboguttata*, *Lepidobatrachus laevis*, *Neobatrachus aquilonius*) survive long-lasting droughts by excavating an underground burrow, constructing a waterproof cocoon of shed skin and mucus, and conserving energy by entering aestivation (Flanigan and Guppy, 1997; Withers, 1995). In this capacity, these frogs are quiescent and immobile in their burrows, often for many months if not years. Consequently, the green-striped burrowing frog (*C. alboguttata*) has recently been used in our laboratory to study the effects of aestivation on skeletal muscle and bone, and the associated physiological and molecular processes that may confer resistance to musculoskeletal deterioration (Hudson et al., 2004, 2006; Hudson and Franklin, 2002a; Mantle et al., 2009; Reilly et al., 2013; Symonds et al., 2007; Young et al., 2013). Prolonged inactivity associated with aestivation has been shown to have negligible effects on muscle mass, whole-muscle cross-sectional area, myofibre number, *in vitro* force production, bone-bending strength and swimming performance in *C. alboguttata* (Hudson et al., 2004, 2006; Hudson and Franklin, 2002a; Mantle et al., 2009). The apparent absence of musculoskeletal losses in *Cyclorana* is consistent with the patterns reported for periods of disuse in hibernators as discussed above, and it is evident that the responses of muscle and bone to disuse in disparate organisms that undergo dormancy are profoundly different from those of typical models of disuse.

Hibernation and aestivation are usually characterised by metabolic depression to preserve energy stores during prolonged fasting (Carey et al., 2003; Guppy and Withers, 1999). For example, metabolic rate is decreased to ∼5% of euthermic levels in hibernating Alpine marmots (Heldmaier et al., 2004), and to 20–30% of the normal resting rate in aestivating frogs (Kayes et al., 2009; Withers, 1993). As musculoskeletal remodelling is associated with a high metabolic cost, increased rates of remodelling (which often occur with disuse) during dormancy would compete for energy reserves with essential physiological processes, such as cardiorespiratory function. However, increased expression of selected genes and proteins in different cellular pathways has been reported during metabolic depression, suggesting that remodelling does occur in certain organs and tissues (Storey and Storey, 2010). For aestivating or hibernating animals, any degenerative changes to muscle and bone would be disadvantageous during the transition from a dormant to an active state. This is particularly true for aestivating frogs that only have a narrow window of opportunity to feed and breed when the summer rains arrive and before the highly ephemeral waters retreat.

Despite a number of hypotheses proposed in the literature as to how muscle and bone deterioration is inhibited during dormancy (Harlow et al., 2001; Tinker et al., 1998; Wickler et al., 1991), a major hindrance to a complete understanding of the phenomenon is the lack of knowledge of the cellular and molecular pathways that are vital for musculoskeletal sparing, and whether any of these are universal across different forms of dormancy. Recent studies have begun to pinpoint important differences between the responses of hibernating and aestivating animals and typical animal models to immobilisation and inactivity. The following sections will discuss several recent exciting studies that examine physiological, molecular and cellular strategies predicted to be paramount in conserving musculoskeletal integrity during dormancy.

Mechanisms underpinning the inhibition of osteoporosis

The black bear (*Ursus americanus*) has been used to study bone remodelling, as these animals are routinely inactive for up to 6 months during hibernation without suffering losses in trabecular or cortical bone mass (McGee-Lawrence et al., 2015). It has been hypothesised that rates of bone resorption and formation remain stable in hibernating *Ursus* to help prevent calcium imbalance (Donahue et al., 2006). However, previous investigations using serum and histological markers of bone remodelling to investigate this idea provided inconsistent data (Donahue et al., 2006; McGee et al., 2008; Seger et al., 2011), possibly as a result of the effect of decreased renal function on serum markers. A more recent study using more reliable markers of bone turnover [bone-specific alkaline phosphatase (BSALP) and tartrate-resistant acid phosphatase (TRACP)] provided strong evidence that bone resorption and formation are suppressed during hibernation in black bears, in contrast to non-hibernating animals undergoing

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disuse (Fig. 4). These data are supported by histomorphometric measurements (osteoid surface, eroded surface, ratio of osteoid to eroded surface) of trabecular bone in grizzly bears, which exhibited no change between active and hibernating states (McGee-Lawrence et al., 2009; McGee et al., 2008). The maintenance of balanced bone formation and resorption during hibernation is consistent with the ability of hibernators to retain calcium homeostasis and, thus, cellular function.

Early studies on ground squirrels, golden hamsters and little brown bats provided evidence that, unlike bears, these species undergo bone loss as a consequence of reduced osteoblastic formation and increased bone resorption during hibernation (McGee-Lawrence et al., 2008). Although these studies lacked thorough quantification of a wide array of bone properties, for small mammalian hibernators there is still conflicting evidence as to whether bone loss regularly occurs during dormancy. Whereas both arctic and golden-mantled ground squirrels retain cortical bone properties and strength following hibernation (Utz et al., 2009; Wojda et al., 2012), data indicate that hibernating *I. tridecemlineatus* might be unable to avoid microstructural losses of cortical and trabecular bone (McGee-Lawrence et al., 2011). Nevertheless, because bone geometrical and mechanical properties do not differ significantly between hibernating and active squirrels, these animals can still perhaps activate the appropriate physiological mechanisms to maintain bone macrostructure and strength throughout hibernation in preparation for normal skeletal function during arousal.

What are the specific mechanisms by which hibernators might largely preserve skeletal function during prolonged inactivity? Despite the knowledge that sclerostin plays an important role in bone mechanotransduction in the majority of mammals, very little is known about sclerostin and related pathways during dormancy. Seger et al. (2011) measured serum sclerostin levels in both hibernating and non-hibernating black bears, and found that there was no significant difference between the two groups. However, a subset of hibernating bears did show increased levels of serum sclerostin, which is a skeletal signal of unloading. Whether these findings translate to a physiological effect in the bone of hibernating bears is unclear.

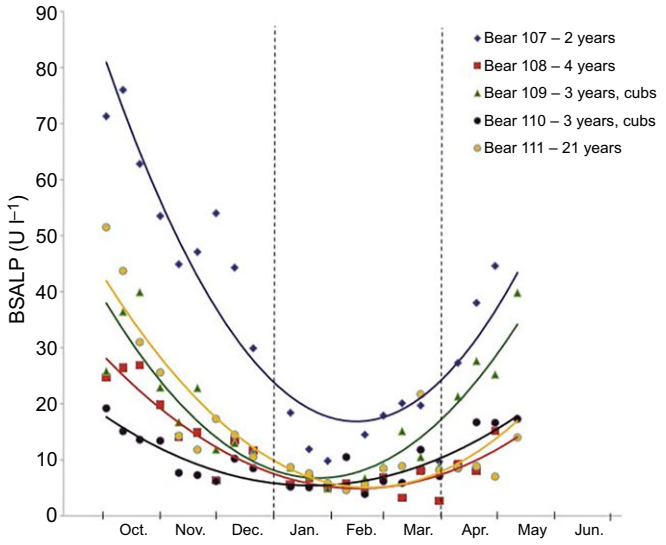

Fig. 4. The concentration of bone-specific alkaline phosphatase (BSALP), a marker of bone formation, measured in sera of black bears (*Ursus americanus*) over one full hibernation season. Dashed lines signify the approximate start and finish times of hibernation (January to March). Levels of serum BSALP were lowest between January and March, demonstrating that hibernating bears have suppressed bone turnover relative to that of active animals (McGee-Lawrence et al., 2015). Reproduced with permission from Journal of Experimental Biology.

As previously mentioned, leptin plays a critical role in energy metabolism by modulating appetite and body mass, but it is also an important effector of sympathetic input into bone through its interactions with brain-derived serotonin. Thus, leptin could contribute to the control of balanced bone remodelling during hibernation and aestivation. In hibernators, serum leptin usually peaks during the autumn as a result of increased food intake in preparation for dormancy (Doherty et al., 2014). Elevated concentrations of leptin during pre-hibernation and early hibernation have been documented in species such as bears, woodchucks, marmots and ground squirrels (see Doherty et al., 2014, and references therein), before the level of leptin gradually drops well into the hibernation season. It has been suggested that suppressed bone resorption in hibernating black bears may be due to leptin-mediated stimulation of hypothalamic CART (Seger et al., 2011). Interestingly, McGee-Lawrence et al. (2015) recently reported a >1000% increase in serum CART levels in hibernating bears compared with pre-hibernation animals, which corroborates the idea that CART may have a potent inhibitory effect on bone resorption throughout dormancy.

At the molecular level, gene expression data from the bone of bears has shown that the transcription of genes involved in anabolic processes of tissue formation (such as cartilage development and osteogenesis) increases during hibernation (Fedorov et al., 2012). Although the expression of genes with known roles in bone resorption was found to be unchanged, Fedorov et al. (2012) reported significant downregulation of genes involved in the stimulation of osteoclast differentiation; it was suggested that this might counteract the increased osteoclastogenesis and bone resorption typically seen in disuse scenarios.

Unfortunately, small- and medium-sized hibernators as well as non-mammalian vertebrates that experience dormancy (aestivating frogs and hibernating snakes, etc.), are extremely understudied with regard to the physiological and molecular mechanisms underpinning the retention of bone properties despite disuse. There are many neural, endocrine, paracrine and mechanical signals that can influence bone mass and strength, and the studies outlined above do offer novel insights into the bone remodelling processes that hibernating bears use to avoid the negative consequences of prolonged disuse. An intriguing question is whether other dormant animals with different strategies of metabolic depression (e.g. hibernating squirrels or bats, and aestivators) demonstrate similar alterations in sclerostin, leptin or CART during dormancy.

Mechanisms underpinning inhibition of muscle disuse atrophy

Examinations of skeletal muscle changes (or lack thereof) in mammalian hibernators have largely focused on rodents, bears and bats, with fewer studies investigating skeletal muscle changes in aestivating amphibians (Cotton, 2016; Hudson and Franklin, 2002b; Y. Maistrovski, Regulation of anti-apoptotic pathways in skeletal muscle and liver of an estivating species, *Xenopus laevis*, MSc thesis, Carleton University, Ontario, Canada, 2011). The morphological changes and remodelling of contractile performance that occur in the muscle of hibernating mammals and aestivating frogs have recently been extensively reviewed elsewhere (Cotton, 2016; James, 2010). Studies on muscle types from small hibernators such as hamsters, ground squirrels and bats have shown relatively small changes in both muscle mass and protein content during

hibernation, with most reporting up to only 15% and 30% decreases in mass and protein, respectively (Cotton, 2016). Alterations in muscle protein content from hibernating bears have been shown to be similar to those of small mammalian hibernators, with an average decrease in muscle protein content of 5–10% (Cotton, 2016). In *C. alboguttata*, 6–9 months of aestivation was reported to have no effect on muscle mass, water content or myofibre number in gastrocnemius or cruralis muscles (Mantle et al., 2009). Similarly, 6 months of aestivation had no effect on the total cross-sectional area of cruralis muscle (Hudson et al., 2006; Mantle et al., 2009). Furthermore, aestivation for 9 months in this species had no effect on myofibre cross-sectional area in the gastrocnemius, a key muscle involved in power output during locomotion (Mantle et al., 2009). Unlike *C. alboguttata*, just 2 weeks of hindlimb immobilisation in more typical laboratory models, such as rats, can promote a 32% reduction in gastrocnemius myofibre cross-sectional area (Sakakima et al., 2004).

Given the overall maintenance of muscle morphological parameters during hibernation and aestivation, it is not surprising that there are also limited changes in muscle mechanics during simulated sprint or endurance-type activities (Cotton, 2016; James, 2010). Below, we discuss several recent studies on multiple species that have addressed the physiological and cellular mechanisms by which dormant animals might resist muscle disuse atrophy and maintain muscle performance.

Regulation of protein synthesis

Under typical conditions, muscle homeostasis is accomplished by balancing the continuous processes of protein degradation and synthesis, which are controlled by mechanisms incorporating multiple signals including energy availability and mechanical loading. Rates of both protein degradation and synthesis can be reduced by up to 70% in the skeletal muscle of hibernating bears (Lohuis et al., 2007). However, more recent studies found increased expression of mRNAs largely involved in protein biosynthesis and ribosome biogenesis in the muscles of both hibernating bears and arctic ground squirrels relative to those of summer active animals, suggesting that induction of translation could be enhanced at different stages of hibernation (Fedorov et al., 2014, 2009). Despite the need for global suppression of energetically expensive processes such as transcription and translation during dormancy (Storey and Storey, 2010), a number of recent studies have emphasised the importance of maintaining protein synthesis in hibernating muscle through activation of the mTOR signalling cascade (Andres-Mateos et al., 2013; Fedorov et al., 2014; Lee et al., 2010; Nowell et al., 2011). For example, hibernating *I. tridecemlineatus* were recently shown to exhibit increased expression of genes in the IGF1–Akt–mTOR signalling pathway (*igf1*, *igf2*, *akt1*, *mTOR* and *rps6kb1*), which is associated with the hypertrophic response to increased mechanical loading and is a key pathway promoting protein synthesis (Vermillion et al., 2015). Interestingly, genes encoding IGF-binding proteins, which have known functions in inhibiting protein synthesis, have also been reported to be strongly suppressed in the skeletal muscle of hibernating squirrels and aestivating frogs (Reilly et al., 2013; Vermillion et al., 2015).

Furthermore, although bats have been found to show depressed rates of protein turnover during torpor, periodic arousals in both bats and squirrels seem to be associated with fluctuations in the activation of mTOR (Lee et al., 2010; Wu and Storey, 2012; Yacoe, 1983). Like mTOR, p70S6 kinase and 4E-BP1 are downstream targets of Akt and are thus key regulatory proteins involved in translation and protein synthesis. The levels of the phosphorylated forms of these proteins increase significantly during hibernation in *I. tridecemlineatus*, which is a strong indicator of activation of mTOR-mediated protein synthesis (Andres-Mateos et al., 2013). The same study showed that increased mTOR activity appears to be controlled by the serum and glucocorticoid-inducible kinase 1 (SGK-1), a previously unknown mediator of skeletal muscle homeostasis and function.

Very recent evidence for increased protein synthesis during hibernation comes from a study that used MRI to measure hindlimb muscle volume, and the relatively novel non-isotopic SUnSET technique to quantify skeletal muscle protein synthesis in ground squirrels (Hindle et al., 2015). It was shown that during early hibernation (October), ground squirrels experienced a 66% decline in protein synthesis compared with fasted animals in the summer. Remarkably, protein synthesis increased significantly as late winter approached and then returned to summer baseline levels, providing strong evidence for regrowth of skeletal muscles during hibernation. Therefore, although increasing protein synthesis is at odds with the need to conserve energy during metabolic depression, a substantial body of evidence suggests that increasing protein synthesis could be necessary at specific time points during dormancy.

Because of its involvement in the regulation of muscle mass, myostatin has been investigated in animals that undergo metabolic depression. Myostatin protein levels in mixed hindlimb muscle of *I. tridecemlineatus* were found to be largely constant throughout torpor relative to those of controls, but increased significantly during arousal (Brooks et al., 2011). In another study on hibernating squirrels, a significant reduction in myostatin gene expression was found in soleus and diaphragm muscles, which are both resistant to atrophy in this species (Nowell et al., 2011). Other studies have shown reduced levels of myostatin mRNA during hibernation and aestivation, which could be associated with enhanced protein synthesis by facilitating mTOR signalling (Reilly et al., 2013; Vermillion et al., 2015).

Regulation of apoptosis

Apoptotic pathways balance signals which either promote or prevent cell death (i.e. pro-apoptotic and anti-apoptotic pathways, respectively). Changes in apoptotic proteins might be expected to occur during hibernation and aestivation in order to preserve cells under conditions that are highly detrimental to most animals (e.g. alterations in temperature, deprivation of nutrients, ischaemia and prolonged muscle inactivity). There is evidence that upregulation of anti-apoptotic mechanisms might be important in maintaining skeletal muscle in aestivating vertebrates (Table 4).

For example, although the African clawed frog (*Xenopus laevis*) is almost exclusively aquatic, this species may enter aestivation during dry periods when water becomes scarce and migration becomes impossible. The transcription factor STAT5, which targets genes that include those with cell survival and proliferation functions, appears to be activated in response to dehydration stress in the skeletal muscle of *Xenopus*, where it probably promotes increased transcription of multiple anti-apoptotic genes and/or proteins (Y. Maistrovski, Regulation of anti-apoptotic pathways in skeletal muscle and liver of an estivating species, *Xenopus laevis*, MSc thesis, Carleton University, Ontario, Canada, 2011) (Table 4). A gene expression profiling study of muscle from aestivating *C. alboguttata* found a coordinated upregulation of genes associated with cell death and survival and DNA replication, recombination and repair (Reilly et al., 2013) (Table 4). The increased expression of a number of pro-apoptotic genes in muscle from aestivating frogs may serve to eliminate individual myocytes (and intracellular

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Table 4. Apoptotic pathways implicated in skeletal muscle remodelling during dormancy

	Species	Summary	Signalling	References
Hibernation	Arctic ground squirrel (*Spermophilus parryii*)	*bcl-2* expression ↑, arousal bouts	Anti-apoptotic	Yan et al., 2008
		birc2 expression ↑, arousal bouts	Anti-apoptotic	
		bid expression ↑, arousal bouts	Pro-apoptotic	
		tp53 expression ↓	Pro-apoptotic	
	Thirteen-lined ground squirrel (*Ictidomys tridecemlineatus*)	Mitochondrial-associated pro-survival proteins ↔	Anti-apoptotic	Rouble et al., 2013
		Birc4 protein ↑	Anti-apoptotic	
Aestivation	Green-striped burrowing frog (*Cyclorana alboguttata*)	*fas* expression ↑	Pro-apoptotic	Reilly et al., 2013
		rassf1 expression ↑	Pro-apoptotic	
		aifm2 expression ↑	Pro-apoptotic	
		birc5 expression ↑	Anti-apoptotic	
		vopp1 expression ↑	Anti-apoptotic	
		htatip2 expression ↑	Anti- or pro-apoptotic	
	African clawed frog (*Xenopus laevis*)	*birc2* expression ↑	Anti-apoptotic	Maistrovski, 2011*
		bcl-xl expression ↑	Anti-apoptotic	
		bcl-2 expression ↑	Anti-apoptotic	
		Birc2 protein ↑	Anti-apoptotic	
		Bcl-xl protein ↑	Anti-apoptotic	
		Bcl-2 protein ↑	Anti-apoptotic	
		Stat5 protein ↑	Anti-apoptotic	
	West African lungfish (*Protopterus annectens*)	TUNEL assay ↑	Pro-apoptotic	Amelio et al., 2013
		Nol3 protein ↓	Anti-apoptotic	

*Y. Maistrovski, Regulation of anti-apoptotic pathways in skeletal muscle and liver of an estivating species, *Xenopus laevis*, MSc thesis, Carleton University, Ontario, Canada, 2011.

macromolecules) that are defective, damaged or otherwise pose a potential threat to the integrity of the animal. Indeed, data from aestivating lungfish indicate increased cellular turnover in skeletal muscle tissue due to activation of apoptotic pathways (Amelio et al., 2013) (Table 4). However, in response to potential apoptotic signals, cells would need to avoid triggering premature or excessive apoptosis, which can be accomplished by balancing the ratio of pro- and anti-apoptotic mechanisms. Accordingly, aestivating burrowing frogs exhibited enhanced expression of genes with reported anti-apoptotic functions [e.g. the inhibitor of apoptosis protein (IAP), also known as survivin or BIRC5; Reilly et al., 2013]. Similar to aestivating frogs, studies of differential gene expression in arctic ground squirrels (*Spermophilus parryii*) found that the mRNA expression of genes involved in apoptosis increased significantly in skeletal muscle during interbout arousals (Yan et al., 2008). In skeletal muscle of ground squirrels, however, the protein expression of xIAP (aka BIRC4) increased significantly in torpid animals (Rouble et al., 2013) (Table 4). This indicates the possibility that IAP-mediated inhibition of caspase activity may be a critical regulatory mechanism which enhances survival of myocytes in dormant species that are resistant to disuse atrophy. These molecular studies are supported at a morphological level, as muscles from hibernating edible dormice exhibit no evidence of necrosis and very few apoptotic myonuclei (Malatesta et al., 2009).

Conclusions and future directions

In this Review we have summarised some of the recent studies that have advanced our knowledge regarding the strategies that might be used by dormant animals to preserve musculoskeletal integrity. By examining the differences and similarities in musculoskeletal biology between different species that undergo distinct forms of dormancy, it is hoped that this Review will promote new insights and ideas for future research.

Global gene expression profiling studies have greatly advanced our knowledge regarding the transcriptional changes that occur in bone and muscle during hibernation and aestivation. These analyses provide an array of candidate genes for future gene or protein expression studies in bone and muscle of dormant animals. The interpretation of the gene expression data is based on the assumption that observed mRNA levels correlate with protein abundance and carry over to a phenotypic response. However, it is probably true that muscle or bone mRNA and protein abundance are not always highly correlated, as a result of regulation at different levels (e.g. post-transcriptional or post-translational regulation). For example, although expression of the *birc5* gene (survivin) was reported to be significantly increased in muscle during aestivation in *C. alboguttata*, it is known that survivin expression is regulated by a number of distinct post-transcriptional mechanisms (Zhang et al., 2006). High-throughput or 'omics' methods, such as proteomic and metabolomic measurements, can provide a more comprehensive assessment of the subcellular changes that may occur in musculoskeletal tissues of hibernators and aestivators, particularly at distinct time points, and how these changes relate to musculoskeletal sparing. Indeed, there were no proteomic signatures of skeletal muscle atrophy (e.g. protein turnover) detected in skeletal muscle of *I. tridecemlineatus* throughout different phases of hibernation (Hindle et al., 2011).

Despite the scale and novelty of information generated using genomics and proteomics techniques, hypothesis-driven studies that test predictions at the whole-animal, tissue, cellular and molecular level will continue to be critical for future investigations of the mechanisms of musculoskeletal sparing during dormancy. For example, while pro- and anti-apoptotic molecules vary in expression and/or abundance in dormant muscle, are there losses in the number of myonuclei during dormancy or does the number of myonuclei not differ from that of active animals?

In future investigations, it would be informative to examine the effect of experimental immobilisation on the skeletal muscles of both non-dormant (i.e. awake, aroused, summer euthermic) and dormant animals of the same species. If dormancy is an important factor in protecting animals against disuse-associated muscle and

bone loss, artificially immobilised limbs of animals experiencing metabolic depression should be less prone to atrophy than immobilised limbs from non-dormant animals. It will also be important to investigate the role of neural mechanisms in musculoskeletal sparing in dormant animals; though we are confident that *C. alboguttata* is inactive and immobile once the cocoon of shed skin has been formed during deep aestivation, it cannot be ruled out that regular neural activation of muscles might be utilised to protect the musculoskeletal system against disuse-induced atrophy. However, hibernating bears were reported to be unusually resistant to the atrophic effects of denervation, indicating that neural activation and/or neural-associated trophic factors may not play a significant role in maintaining muscle architecture during hibernation (Lin et al., 2012). A similar type of response to loss of neuromuscular communication might be envisaged for aestivators, given that the quantity of acetylcholine neurotransmitter 'packages' (i.e. quanta) released per synapse is reduced in 6 month aestivating *C. alboguttata* (Hudson et al., 2005).

To further our understanding of disuse-induced musculoskeletal loss across vertebrates, and for the possible development and authentication of new therapies, the use of suitable animal models continues to be paramount. Although hibernators and aestivators could be developed into model organisms in this area of biomedical research, a greater understanding of their biological 'toolbox' is required. This would include the construction of genomes from a range of species, an extensive catalogue of their genes and genetic variation, characterisation of different proteomes and transcriptomes, and the development of bone and muscle cell lines. Hibernators and aestivators are fascinating study systems that can provide useful insights into the regulation of musculoskeletal integrity during prolonged disuse, furthering our understanding of both the basic underlying controls and the responses of the musculoskeletal system to specific challenges. The common molecular features and variety of pathways at play during hibernation and aestivation will hopefully provide new directions for future studies of metabolic suppression and the associated preservation of muscle and bone.

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