ABSTRACT

Mobilization of body reserves including fat, protein, and glycogen is necessary to overcome phases of negative nutrient balance typical for high-yielding dairy cows during the periparturient period. Skeletal muscle, the largest internal organ in mammals, plays a crucial role in maintaining metabolic homeostasis. However, unlike in liver and adipose tissue, the metabolic and regulatory role of skeletal muscle in the adaptation of dairy cows to the physiological needs of pregnancy and lactation has not been studied extensively. The functional integrity and quality of skeletal muscle are maintained through a constant turnover of protein, resulting from both protein breakdown and protein synthesis. Thus, muscle protein breakdown (MPB) and synthesis are intimately connected and tightly controlled to ensure proper protein homeostasis. Understanding the regulation of MPB, the catabolic component of muscle turnover, and its assessment are therefore important considerations to provide information about the timing and extent of tissue mobilization in periparturient dairy cows. Based on animal models and human studies, it is now evident that MPB occurs via the integration of 3 main systems: autophagy-lysosomal, calpain Ca²⁺-dependent cysteine proteases, and the ubiquitin-proteasome system. These 3 main systems are interconnected and do not work separately, and the regulation is complex. The ubiquitin-proteasomal system is the most well-known cellular proteolytic system and plays a fundamental role in muscle physiology. Complete degradation of a protein often requires a combination of the systems, depending on the physiological situation. Determination of MPB in dairy cows is technically challenging, resulting in a relative dearth of information. The methods for assessing MPB can be divided into either direct or indirect measurements, both having their strengths and limitations. Available information on the direct measures of MPB primarily comes from stable isotopic tracer methods and those of indirect measurements from assessing expression and activity measures of the components of the 3 MPB systems in muscle biopsy samples. Other indirect approaches (i.e., potential indicators of MPB), including ultrasound imaging and measuring metabolites from muscle degradation (i.e., 3-methylhistidine and creatinine), seem to be applicable methods and can provide useful information about the extent and timing of MPB. This review presents our current understanding, including methodological considerations, of the process of MPB in periparturient dairy cows. Key words: muscle, tissue mobilization, transition period, dairy cow

MUSCLE PROTEIN BREAKDOWN DURING THE PERIPARTURIENT PERIOD

The periparturient period in dairy cows, defined as 3 wk before until 3 wk after calving (Grummer, 1995), is crucial for health and production; it is characterized by comprehensive changes in metabolic, endocrine, and immune functions (Drackley, 1999). Insufficient nutrient intake during the periparturient period (Hayirli et al., 2002; Kuhla et al., 2011) and increased nutrient demand for fetus and mammary tissue growth in late gestation and colostrum and milk synthesis at the onset of lactation commonly result in a negative nutrient balance. Thus, mobilization of body reserves, including fat, protein, and glycogen, is necessary to overcome phases of negative nutrient balance (Ingvartsen and Andersen, 2000). The degree of body weight loss during the periparturient period seems to be an intrinsic trait in high-yielding cows (Zachut et al., 2013; Zachut and Moallem, 2017), likely with a genetic basis (Friggins et al., 2007). The success of adaptation to these physiological changes is highly variable among individual cows, and compromised adaptation may result in production diseases (Drackley, 1999; Sundrum, 2015). The metabolic and regulatory roles of adipose tissue have been extensively studied during the past decade. In contrast,
the mobilization of body protein in dairy cows during the periparturient period has been less investigated. The bulk of mobilized protein seems to be derived from peripheral tissues, in particular skeletal muscle and, to a lesser extent, skin (Meijer et al., 1995). Thus, skeletal muscle, with the greatest contribution to whole-body protein turnover because of its large protein mass (Frayn, 2010), plays a major role in maintaining metabolic homeostasis and adaptation to the physiological needs of pregnancy and lactation (Phillips et al., 2003; Kuhla et al., 2011; Ji and Dann, 2013). The underlying mechanisms regulating peripartum muscle protein breakdown (MPB) may be mediated by endocrine changes, including hypoinsulinemia and diminished muscle responsiveness to insulin, growth hormone, and IGF-1 concentrations (Bell et al., 2000; De Koster and Opsomer, 2013), suppression of tissue protein synthesis, and upregulated expression of components of proteolytic pathways in the muscle (e.g., Chibisa et al., 2008; Greenwood et al., 2009; Mann et al., 2016; Ghaffari et al., 2019; Yang et al., 2020).

Estimates of the amount of body protein loss in periparturient dairy cows range from approximately 8 to 21 kg (Komaragiri and Erdman, 1997; Komaragiri et al., 1998; Phillips et al., 2003; Chibisa et al., 2008). Mobilization of body protein reserves seems to be restricted, with repletion occurring by wk 5 postpartum, whereas body fat mobilization continues for an extended period up to 12 wk postpartum (Komaragiri and Erdman, 1997; Komaragiri et al., 1998). However, besides the level of production and dietary regimen, the technique applied to measure body protein mobilization may lead to variations in results. Interestingly, MPB in most cows seems to start antepartum, even before the onset of lipid mobilization, perhaps due to a prepartum AA deficiency even when energy balance is neutral or positive (van der Drift et al., 2012; Mann et al., 2016; Yang et al., 2020). Furthermore, MPB in early lactation does not seem to be a function of intake and dietary factors, but rather a function of milk yield and the endocrine status associated with lactation (Bauman and Currie, 1980; Komaragiri and Erdman, 1997; Komaragiri et al., 1998; Overton, 2001; Phillips et al., 2003).

Given the increasing demand for glucose in postpartum dairy cows, it has been hypothesized that this need is met by increased utilization of glucogenic AA for liver gluconeogenesis (Overton, 1998; Drackley et al., 2001), and these AA indeed contribute to the lacking glucogenic carbon (Danfær et al., 1995). It is thus believed that MPB after parturition may, to a certain extent, provide AA for gluconeogenesis and thus limit ketone body production. However, this concept has been challenged by quantitative investigations using splanchnic-catheterized periparturient dairy cows (Reynolds et al., 2003; Larsen and Kristensen, 2009). A review of available quantitative data on hepatic metabolism of glucose, AA, and other glucogenic precursors in the periparturient period does not support the notion that the rapid postpartum increase in net liver release of glucose and concurrent lack of glucogenic VFA (i.e., propionate, isobutyrate, and valerate; Bergman, 1990) are counterbalanced by increased utilization of AA for gluconeogenesis (Larsen and Kristensen, 2013). Larsen and Kristensen (2013) concluded that a vast majority of the glucose derived during early lactation is from the endogenous recycling of glucogenic carbon through lactate and only Ala is likely to contribute to liver release of glucose, through its role in the interorgan transfer of nitrogen from catabolized AA. This challenges the concept that AA are significant contributors to liver gluconeogenesis in in postpartum dairy cows. Thus, AA derived from muscle proteolysis after parturition seem to be prioritized for anabolism, allowing a greater amount of AA for milk synthesis (Lapierre et al., 2012; Larsen and Kristensen, 2013; Larsen et al., 2015).

During the peripartal period, high-yielding dairy cows generally experience a period of immune dysfunction (Kehrli et al., 1989a,b; Kimura et al., 1999; Nonnecke et al., 2003; Goff, 2006; Sordillo et al., 2009; Sordillo, 2016). In most species, protein deficiency is associated with significant impairments of immune functions. The need for certain nutrients, such as AA, is likely not covered by feed intake or mobilization from tissues, and may result in “hidden” nutrient deficiencies that are not taken into account in the calculation of nutrient requirements (Ji and Dann, 2013). The potential requirement for protein and AA to support the immune system concerns proliferation of immune cells, as well as production of cytokines, antibodies, and effector molecules, that may add more burden to breakdown of muscle tissue (Ji and Dann, 2013). In addition, the acute phase response (APR) is considered a more intensive proteinaceous process than activation of immune cells alone (Klasing, 1998). The AA composition of skeletal muscle is not a very good match for acute phase proteins (APP), integral parts of the APR, indicating the need for overmobilization of body protein to meet the demand of specific AA for APP synthesis (Reeds et al., 1994). The concentrations at which APP occur in the circulation are relatively high; the 2 major positive APP in cattle—that is, haptoglobin and serum amyloid A—may increase by more than 10-fold during the APR, reaching double-digit micrograms per milliliter (serum amyloid A) and milligrams per milliliter (haptoglobin) ranges, and thus requiring substantial AA supply (Ceciliani et al., 2012). Elevated concentrations

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of APP are known to accompany parturition in cows (e.g., Saremi et al., 2012 for haptoglobin; Meglia et al., 2005 for serum amyloid A). However, the concentrations of negative APP (serum proteins such as albumin that decrease in concentration during the acute phase in response to inflammation or infection) that are decreased during APR might attenuate this need for AA. The mechanism for the decrease in the concentrations of negative APP is not clear, but is likely multifactorial, including reduced liver production in response to inflammatory cytokines, and probably increased loss or increased proteolysis (Ceciliani et al., 2012). Reduced production of negative APP by the liver or release of AA after proteolysis of negative APP might allow greater increase in the amount of AA available for positive APP production. Amino acids can be used as fuel by the immune system, either directly or following their conversion to other AA (e.g., Gln) or to glucose (Li et al., 2007; Kelly and Pearce, 2020). Catabolic states are associated with increased susceptibility to infections (Calder, 2006). This may be related to suboptimal supply of substrates, including AA, to the immune system, as a critical role of various AA in regulating different steps of both innate and adaptive immunity has been documented (Grohmann and Bronte, 2010; McGaha et al., 2012). Thus, in view of the substantial mobilization of muscle protein during the transition period and the specific requirements of AA for the immune system, the latter should be considered when setting requirements in feeding systems.

**SYSTEMS OF MUSCLE PROTEIN BREAKDOWN**

Muscle protein turnover is a dynamic and continual process of protein synthesis and breakdown. The balance between the rates of protein breakdown and synthesis (i.e., net muscle protein balance) determines the muscle protein pool (mass). Thus, these 2 processes are intimately connected and tightly controlled to ensure proper protein homeostasis. Three main systems contribute to the catabolic component of muscle protein turnover: autophagy-lysosomal, calpain Ca\(^{2+}\)-dependent cysteine proteases, and the ubiquitin-proteasomal system (UPS). The contribution of each system to protein breakdown in healthy muscle is dependent on endogenous and exogenous factors, including metabolic and nutritional (e.g., AA, energy) as well as hormonal (e.g., insulin) status (Pasiakos and Carbone, 2014). These 3 interconnected main systems do not work in isolation but rather work together to remodel skeletal muscle (Tipton et al., 2018). Schematic overviews of the 3 main systems that contribute to the catabolic component of muscle protein turnover are presented in Figures 1 and 2.

**Autophagy-Lysosome**

Autophagy, a highly conserved intracellular proteolytic homeostatic mechanism, plays a vital role in the degradation and recycling of bulk cytoplasmic, durable proteins, and organelles through the lysosomal machinery (Mizushima and Komatsu, 2011). There are 3 primary types of autophagy in mammals, including microautophagy, macroautophagy, and chaperone-mediated autophagy, and they differ in their modes of delivery of cytoplasmic cargo to the lysosome for degradation (Parzych and Kliionsky, 2014). Microautophagy involves the direct uptake of cytosolic cargo by invagination of the lysosomal membrane (Parzych and Kliionsky, 2014). Chaperone-mediated autophagy is highly specific and translocates substrate proteins directly across the lysosomal membrane for degradation. During chaperone-mediated autophagy, the specific pentapeptide motif of the substrates, called the KFERQ motif, is recognized by 70 kDa heat shock cognate protein 8 (HSPA8/HSC70), as well as other chaperones (Tasset and Cuervo, 2016). Unlike microautophagy and chaperone-mediated autophagy, macroautophagy involves sequestration of the cargo away from the membrane of the lysosome. It relies on de novo synthesis of cytosolic double-membrane vesicles, autophagosomes, to sequester cargo and subsequently transport it to the lysosome (forming an autophagolysosome; Parzych and Kliionsky, 2014). All 3 types of autophagy lead to the degradation of cargo, and the resulting breakdown products are sent back into the cytosol for reuse by the cell.

Of the 3 types of autophagy, most data in muscle are related to macroautophagy; however, whether microautophagy and chaperone-mediated autophagy have roles in muscle homeostasis or atrophy is not yet well understood (Bonaldo and Sandri, 2013). Cathepsins, as the most abundant lysosomal proteases, are also involved in degradation of muscle proteins with specificity toward particular myofibrillar substrates (e.g., troponin T, myosin heavy chain, and tropomyosin; Bechet et al., 2005). In addition, the autophagy-lysosomal system is considered to be particularly important for degrading membrane-based proteins (e.g., transporters, ion channels, and receptors; Mayer, 2000). With regard to their importance in the control of anabolic processes, regulation of the degradation of membrane-based proteins has a crucial role in muscle remodeling (Tipton et al., 2018). Fast-twitch muscle fibers exhibit lower cathepsin activities than slow-twitch muscle fibers, although expression of cathepsins is generally low in skeletal muscle (Pasiakos and Carbone, 2014). Autophagy-lysosomal proteolysis has been shown to be increased by a plethora of situations including fasting in rodents (Mamucari et al., 2007; Mizushima et al., 2004; Sandri,
Figure 1. A schematic overview of the 3 main systems that contribute to the catabolic component of muscle protein turnover. (A) Macroautophagy: the phagophore, or isolation membrane (1), becomes a cup-shaped double membrane structure and curves until its ends merge to sequester portions of the cytoplasm, resulting in the formation of an autophagosome (2). The outer membrane of the autophagosome is fused with the lysosomal membrane, forming an autophagolysosome (3). The sequestered cellular components and the inner membrane of the autophagosome are degraded by the lysosomal hydrolases (4). The resulting breakdown products are sent back into the cytosol for reuse by the cell (5). (B) Calpain Ca$^{2+}$-dependent cysteine proteases: calpains are activated by Ca$^{2+}$ (the most important activator), although other mechanisms including autolysis may also contribute to calpain activation. Calpastatin is a calpain-specific endogenous inhibitor. Calcium also influences the binding of calpastatin to calpain, resulting in inhibited calpain activity. Calpains are responsible for limited proteolytic events, which lead to the regulation of the activity of their substrates rather than their degradation. (C) Ubiquitin-proteasomal system: an E1 enzyme activates ubiquitin (Ub) in an ATP-dependent manner and transfers it to an E2 ubiquitin-conjugating enzyme. An E3 ubiquitin ligase subsequently recruits the target protein and mediates the transfer of ubiquitin to the protein, leading to ubiquitination of the target protein. The 26S proteasome enzyme complex recognizes poly-Ub as the main tag; the targeted proteins thus must be labeled with at least 4 Ub monomers. The 26S proteasome is composed of a 20S catalytic core and two 19S regulatory caps. The 19S regulatory cap with a deubiquitinating activity removes and releases Ub into the cytoplasm, and the proteins are linearized and injected to the 20S catalytic core of the proteasome, where it is broken down to peptides.
2010) and AA deprivation, particularly Leu starvation, in cultured myotubes (Mordier et al., 2000), although cathepsin activity decreases with sustained energy deficit (Belkhou et al., 1994). Mann et al. (2016) were the first to identify the potential role of macroautophagy mechanisms of muscle protein proteolysis in periparturient dairy cows. As will be further discussed, they found that macroautophagy was upregulated in postpartum negative energy and protein balance, regardless of dietary energy strategy prepartum, and was greater in cows overfed energy during the periparturient period (Mann et al., 2016).

**Calpain Ca²⁺-Dependent Cysteine Proteases**

Calpains are Ca²⁺-dependent and non-lysosomal cysteine proteases. Three major types of calpains are expressed in the skeletal muscle: calpain-1 (μ-calpain), calpain-2 (m-calpain; the ubiquitous calpains), and the muscle-specific calpain-3 (p94: Sorimachi et al., 1989). The names of 2 isoforms, μ-calpain and m-calpain, refer to their reliance on micro- or millimolar concentrations of Ca²⁺ for activation (Goll et al., 2003). The name of p94 for calpain-3 refers to its larger molecular weight of 94 kDa compared with the 80-kDa mass of calpain-1.

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**Figure 2.** Muscle protein breakdown occurs through the integration of 3 main systems—autophagy-lysosomal, calpain Ca²⁺-dependent cysteine proteases, and the ubiquitin-proteasome system. These 3 main systems are interconnected and do not work in isolation but rather work together, and the regulation is complex. The underlying mechanisms regulating peripartum muscle protein breakdown may be mediated by endocrine changes, including hypoinsulinemia and diminished muscle responsiveness to insulin, growth hormone, and IGF-1 concentrations. The hormones glucocorticoids and glucagon might have catabolic effects and induce muscle protein loss, which should be investigated in future studies in periparturient dairy cows. Figure was created using BioRender (agreement number: SV243ACX47).
and calpain-2. Binding of Ca\(^{2+}\) to 2 Ca\(^{2+}\) binding sites on calpains serves as an essential allosteric regulator, leading to a conformational change in the cysteine protease region of calpain and subsequent activation (Hyatt and Powers, 2020). Calpastatin, a highly polymorphic protein, is a calpain-specific endogenous inhibitor (Goll et al., 2003), which—in addition to calcium—plays a central role in the regulation of calpain activity (Dargelos et al., 2008). The expression level of calpastatin has been shown to be regulated by genetics and nutrients (Geesink and Koohmaraie, 1999; Helman et al., 2003). Calpain-3 is characterized by a rapid and complete autolysis, resulting in its disappearance from muscle (Sorimachi et al., 1993; Kinbara et al., 1998). Myofibrillar, cytoskeletal, and sarcolemmal proteins are known as protein target substrates for calpain activity in muscle. Calpain-3 seems to be mainly bound to myofibrillar proteins and in particular to titin (Murphy and Lamb, 2009) and plays an important role in skeletal muscle homeostasis (Saenz et al., 2005). Calpains seem to have limited capability to fully degrade complex intramuscular substrates (Costelli et al., 2005); however, the calpain proteases disassemble myofibrillar proteins into smaller component parts, the mechanism by which calpains likely contribute to muscle proteolysis (Goll et al., 2003). This initial calpain-mediated cleavage consequently increases substrate accessibility to the UPS for degradation into individual AA (Costelli et al., 2005). As will be further discussed, alterations in calpastatin content in the muscle of nutrient-restricted Angus-Gelbvieh crossbred cows (Du et al., 2004b), as well as calcium-dependent calpains mRNA in the muscle of periparturient dairy cows (Chibisa et al., 2008), have been reported.

**Ubiquitin-Proteasomal System**

The UPS, the best-known protein breakdown system, is regarded as the central proteolytic pathway in the muscle and plays a fundamental role in normal physiology (Kitajima et al., 2020). The controlled degradation of individual proteins mediated by the UPS is also crucial in maintaining free AA pools when AA supply is disrupted (Ciechanover, 2005; Cohen-Kaplan et al., 2016). The proteins targeted for degradation by the UPS are first “tagged” in the ubiquitination pathway and are later proteolyzed by the proteasome enzyme complex (Nandi et al., 2006; Bodine and Baehr, 2014). Ubiquitination of proteins requires the coordinated reactions of 3 enzymes, including ubiquitin-activating enzyme (E1), ubiquitin-conjugating enzyme (E2), and ubiquitin ligases. Ubiquitin is first activated by the ATP-dependent E1 enzymes. Once activated, ubiquitin is recognized by the E2 and transferred to the enzyme’s active-site cysteine via transthioesterification (Lee and Schindelin, 2008). The specific E3 ligases are responsible for the selection of the target proteins that should be ubiquitinated and catalyze the transfer of ubiquitin from E2 to a Lys group of the target proteins (Murton et al., 2008). The proteasome enzyme complexes are formed by the combination of a catalytic core particle (also known as the 20S proteasome) with 1 or 2 terminal 19S regulatory particles that serve as a proteasome activator (Nandi et al., 2006; Tanaka, 2009). The proteasome enzyme complex recognizes poly-ubiquitin as the main tag; the targeted proteins thus must be labeled with at least 4 ubiquitin monomers, though noncanonical ubiquitin-based signals for proteasomal degradation have also been reported (Kravtsova-Ivantsov and Ciechanover, 2012).

The 2 muscle-specific E3 ubiquitin ligases, tripartite motif containing 63 (TRIM63; also called muscle-specific ring-finger protein 1, MuRF-1) and Fbox only protein 32 (FBXO32; also called atrogin-1), are considered important components of the UPS and have been the focus of many human and animal studies. These 2 E3 ligases are transcriptionally upregulated together under most atrophy-inducing conditions (Bodine et al., 2001; Gomes et al., 2001; Lecker et al., 2004; Foletta et al., 2011), suggesting that they are under the regulation of similar sets of several transcription factors, including the forkhead transcription factors (FOXO1 and FOXO3a), NF-κB transcription factors (p65, c-Rel, RelB, p52, and p50), Krüppel-like factor 15 (KLF-15), CCAAT/enhancer-binding protein-3 (C/EBPβ), and activation of the glucocorticoid receptor. These transcriptional mediators can bind to the promoter regions of either the MuRF-1 or MAFbx/atrogin-1 genes, resulting in an increase in their expression levels within the muscle (Bodine and Baehr, 2014). As will be further discussed, changes in mRNA or protein expression of the 2 muscle-specific E3 ubiquitin ligases, MuRF-1 and atrogin-1 (Mann et al., 2016; Sadri et al., 2016; Ghaffari et al., 2019; Yang et al., 2020), other components of the UPS including ubiquitin (Chibisa et al., 2008; Greenwood et al., 2009), ubiquitination of proteins (Du et al., 2005; Mann et al., 2016), ubiquitin-conjugating enzyme E2, and proteasome 26S subunit, ATPase (Chibisa et al., 2008; Greenwood et al., 2009; Ghaffari et al., 2019; Yang et al., 2020), as well as 20S proteasome activity (Mann et al., 2016; Yang et al., 2020), have been reported in dairy cows studies.

As stated above, the 3 MPB systems are interconnect ed and do not work separately. The UPS alone is unable to degrade intact myofibrils due to their relatively large size (Solomon and Goldberg, 1996; Du et al., 2004a).
Thus, there is a requirement for involving one or both of the autophagy-lysosomal and Ca-dependent calpains, depending on the physiological situation. In the case of the degradation of sarcomeric proteins, an initial calpain-mediated cleavage into their component parts is thought to be required by the UPS (Jackman and Kandarian, 2004). Although the autophagy-lysosome system had previously been considered a nonspecific degradation system, it was shown to degrade ubiquitinated proteins (Pankiv et al., 2007). A complementary relationship exists between autophagy and UPS (Masiero et al., 2009; Kitajima et al., 2014), and these 2 systems seem to be compensatory to maintain myocellular homeostasis and integrity (Kitajima et al., 2020). For instance, muscle-specific autophagy dysfunction induces an increase in ubiquitinated proteins during denervation, reflecting a compensatory upregulation of the UPS (Masiero et al., 2009). Muscle-specific Rpt3-knockout (proteasome-deficient) mice were found to exhibit activation of autophagy (Kitajima et al., 2014). Moreover, autophagy has been induced in response to impaired UPS activity in different cell lines in vitro (Iwata et al., 2005). These findings suggest a crosstalk between the autophagy and proteasomal pathways in the skeletal muscle.

ASSESSMENT OF MUSCLE PROTEIN BREAKDOWN

A better understanding of muscle protein turnover requires knowledge of both muscle protein synthesis (MPS) and MPB. Due to the complexity associated with the assessment of MPB, limited information is available even from human studies about the influence of nutrition on MPB, as compared with the extensive literature that exists on MPS. Several methods for assessing MPB exist; these can be divided into either direct or indirect measurements, both having their strengths and limitations. Direct measures of MPB can be determined primarily, albeit not entirely, using stable isotopic tracer methods. Indirect measurements of MPB stem primarily from assessing changes at the mRNA or protein level related to the 3 protein breakdown pathways in muscle biopsy samples, which are considered as surrogate markers to describe myofibrillar proteolysis. In addition, the changes in urinary or circulating 3-methylhistidine (3-MH), creatinine, and the ratio thereof have been used to provide a crude estimate of MPB. Ultrasonographic measurement of muscle thickness is also increasingly used as an on-farm method for monitoring MPB in periparturient dairy cows. The deuterium oxide (D₂O) dilution method has also been used in limited studies to estimate body composition and (indirectly) monitor MPB in periparturient dairy cows.

Direct Measures of Muscle Protein Breakdown (Stable Isotopic Tracer Methods)

Most of the available methods for assessing direct MPB involve the use of stable isotopic tracer techniques. These techniques, which require the infusion of stable isotope-labeled AA combined with multiple arteriovenous (AV) blood sampling (with or without muscle biopsy) have been used to assess both MPS and MPB in vivo. The available methods can be classified into 2 groups: AV balance methods (2- and 3-compartment AV tracer models) and precursor-product methods, including the tracee release method, the pulse tracer injection method, and the modified pulse tracer injection method. The AV balance methods pool the entire leg muscle, whereas the precursor-product methods allow measurement of the fractional breakdown rate (FBR) of a specific muscle corresponding to the site of the biopsy. The different precursor-product methods are distinguished by whether or not they require an isotopic steady state or multiple tracers and by how many muscle tissue biopsies are needed. If isotopic steady state measurements are established, then measurements at only 2 points are theoretically needed; if not, then measurements at 3 points are theoretically needed to measure FBR (Chinkes, 2005).

Two-Compartment Arteriovenous Tracer Model

The 2-compartment model (arterial and venous free AA pools), also known as the AV balance model, is used for the calculation of the uptake and release of a labeled AA (such as Phe, which is neither synthesized nor oxidized in muscle) into circulation across the limb, assuming that the uptake and release are due directly to MPS and MPB, respectively (Thompson et al., 1989a,b). Thus, to assess MPB using this model, the outward labeled AA transport from the limb muscle (most commonly the leg and the forearm) into the venous pool is assumed to be equivalent to MPB, and both processes are assumed to be in steady state (Thompson et al., 1989a,b). Thus, concerns about the 2-compartment model arise from underestimating the true rate of MPB depending on the amount of labeled AA that appears in the muscle intracellular pool from MPB, which is reutilized for MPS and not transported out into the venous blood (Tipton et al., 2018; Joanisse et al., 2021). With ruminants the AV technique has been applied to hindlimb studies predominantly in sheep during the fed-fasted cycle (e.g., Pell et al., 1986; Teleni et al., 1986; Oddy et al., 1987; Harris et al., 1992), and some in growing steers (e.g., Boisclair et al., 1993, 1994) and lactating goats (Bequette et al., 2002).
**Three-Compartment Arteriovenous Tracer Model**

In the 3-compartment model developed by Biolo et al. (1992, 1994) and applied to the hind-leg, in addition to arterial and venous blood samples, the isotopic enrichment of intracellular AA tracers is also determined from muscle biopsy samples, thus providing an actual rate of appearance of AA into the muscle intracellular pool from MPB. Thus, the 3-compartment model might give a closer approximation of the true rate of MPB (Tipton et al., 2018; Joanisse et al., 2021). To our knowledge, no study has been published addressing the use of 3-compartment models for estimation of MPB in periparturient dairy cows. The use of stable isotope tracers approaches in ruminants has mainly been focused on studying different AA uptake by the mammary gland and liver of lactating dairy cows (e.g., France et al., 1999; Crompton et al., 2014, 2018) or goats (e.g., Bequette et al., 1994, 1996, 1997, 2000; Hanigan et al., 2009). For instance, a 3-compartment model of mammary gland AA kinetics, based on the model of Biolo et al. (1992), was used to derive rates of transfer of AA from plasma to the intracellular pool and from the intracellular pool to the plasma in lactating cows (Bequette et al., 2000). In a follow-up study on mammary tissue protein turnover in lactating goats from the same team (Hanigan et al., 2009), the authors highlighted a limitation encountered with the Biolo model (Biolo et al., 1992). This model calculates uptake and efflux rates from arterial and venous concentrations and enrichments and does not take an extracellular pool into consideration. Because of the high rate of exchange between vascular and interstitial spaces, Hanigan et al. (2009) suggested that using venous concentrations and enrichments to calculate uptake seems to be more appropriate, as the capillary and interstitial pools will be very close to equilibrium.

**Tracee Release Method**

Zhang et al. (1996) developed a method to measure the FBR of muscle tissue protein. This method involves infusion of an isotope tracer to reach isotopic equilibrium and then measuring its decay in the arterial and muscle intracellular free pools. The FBR is thus calculated based on the rate at which the tracee released from MPB dilutes the intracellular enrichment through a modified precursor-product equation. In this method, the precursor is the protein-bound AA and arterial AA, and the product is the intracellular free AA. This method assumes that a physiological steady state exists and takes around 4 to 5 h to perform, and usually 3 tissue biopsies are required. The FBR measurement can be combined with the primed-constant infusion to assess both fractional synthesis rate (FSR; the rate of incorporation of free AA into protein versus total muscle protein) and FBR (the rate of release of free AA from proteolysis versus total muscle protein) simultaneously in the same infusion study (e.g., Ferrando et al., 1999; Phillips et al., 1999; Brook et al., 2022).

**Pulse Tracer Injection Method**

Zhang et al. (2002) developed a less invasive stable isotopic tracer model to assess both FSR and FBR of muscle tissue protein in vivo within a shorter time frame that does not require arterial catheterization or measuring tissue blood flow. The pulse tracer injection method involves a bolus injection of labeled AA and measurement of enrichment in the arterial blood and muscle biopsies, generating comparable results with the 3-compartment AV model under controlled conditions (Zhang et al., 2002; Miller et al., 2004; Chinkes et al., 2008). This method is based on the principle that appearance of unlabeled AA (e.g., Phe) from MPB will dilute the tracer (e.g., Phe tracer) enrichment in the muscle intracellular pool, but not the arterial blood pool. Thus, the enrichment decay is expressed as tracer-to-tracee ratio, reflecting dilution of the tracer in the muscle intracellular free pool by the unlabeled AA (Phe) released from protein breakdown. The FSR and FBR are calculated based on the precursor-product principle, but with the difference that the definitions of precursor and product are reversed for the 2 processes. In the case of protein synthesis, the muscle intracellular free AA (precisely, aminoacyl-tRNA) is the precursor, and the product is the protein-bound pool. In the case of protein breakdown, the arterial blood and protein-bound AA pool in muscle are the precursors, and the muscle intracellular free AA is the product pool (Zhang et al., 2002). In contrast with the tracee release method, this method does not require measurement at isotopic steady state (if the arterial and intracellular enrichments are measured at 3 time points), FSR and FBR are measured over the same time period (about 1 h), and the dose of tracer does not stimulate MPS (Zhang et al., 1996, 2002). Notably, if multiple tracers are used, then the number of biopsies can be reduced (Chinkes, 2005). Nevertheless, the pulse tracer injection method also assumes the presence of a physiological steady state, in which the concentrations of AA in the blood and in the muscle intracellular free pool are constant. To our knowledge, this method has never been used in dairy cows. The pulse tracer injection method, as a less invasive approach (compared with the AV balance methods) that does not require an isotope plateau and allows measurement of both muscle protein FBR and FSR over a 60-min time frame, should be a subject of
future studies in periparturient dairy cows for measuring muscle tissue protein breakdown rate in vivo.

**Modified Pulse Tracer Injection Method**

Tuvdendorj et al. (2013) modified the pulse tracer injection method to assess FBR of leg muscle protein during a physiological nonsteady state of AA in rabbits. This method involves a bolus injection of unlabeled Phe to develop a physiological nonsteady state of Phe and labeled Phe and Thr (as an independent control tracer) to assess muscle protein FBR. The FBR is calculated based on the precursor-product principle but with the addition of 2 new assumptions that inward transport of AA is proportional to their arterial concentrations and that protein breakdown is constant over the time period when the measurement is performed. To our knowledge, this method has never been validated in humans. Moreover, a bolus injection of unlabeled Phe was used to develop a physiological nonsteady state, and the effect of a meal has not been validated in either animal models or humans.

**Fractional Breakdown Rate of Protein Using D₂O**

In vivo approaches using isotope dilution and arteriovenous catheterization do not permit estimation of the breakdown rate of specific proteins. Holm et al. (2013) developed a method to measure steady-state FBR of individual proteins using administration of D₂O and incorporation of the deuterium into Ala that was subsequently incorporated into body proteins. The rate of disappearance of deuterated Ala from the proteins allowed for quantification of the FBR of proteins. This method was tested in rats, and its applicability has also been demonstrated in humans (Holm et al., 2013).

**Limitations Associated with Direct Measures of Muscle Protein Breakdown**

An important concern about the use of AV balance (2- or 3-compartment) methods is the invasive nature of the methods that require the catheterization of an artery and a deep forearm or femoral vein for multiple sampling throughout an experiment. Thus, using these methods in humans is mostly limited to clinical facilities and not very feasible in studies with healthy volunteers. Because samples are taken from venous blood draining an entire limb (not only the muscle tissue) in the AV balance models, the calculated MPB includes the catabolic contribution from nonmuscle tissues (e.g., skin and bone), though under rest conditions the leg provides a reasonable representation of muscle metabolism (85–90%; Biolo et al., 1994). Both AV balance and FBR methods do not distinguish between the different pathways responsible for MPB, but only allow for measuring the total rate of protein breakdown in the muscle tissue (Chinkes, 2005). Another limitation for the AV balance methods and some of precursor-product methods is that both physiological and isotopic steady states are required to produce robust results. However, in many nutrition studies, a physiological steady state is unattainable, indicating that these methods may not be suitable for quantifying the response to feeding.

**Indirect Measurements of Muscle Protein Breakdown (Studying Molecular Pathways of Muscle Protein Breakdown)**

Indirect measurements of MPB (i.e., potential indicators of MPB), are mainly based on assessing changes at the mRNA or protein level or on indices of protein signaling and activation responses (e.g., phosphorylation, autolysis) within the 3 protein breakdown pathways. Changes in mRNA or protein expression of the 2 muscle-specific E3 ubiquitin ligases, MuRF-1 and atrogin-1, are most commonly measured to assess changes in MPB under various interventions in humans (e.g., Pasiakos and Carbone, 2014), pigs (Suryawan and Davis, 2014), calves (Sadri et al., 2017), and dairy cows (Mann et al., 2016; Sadri et al., 2016; Ghaffari et al., 2019; Yang et al., 2020). The muscle-specific E3 ubiquitin ligases determine the substrate specificity of the UPS-dependent proteolysis and are upregulated during muscle-wasting conditions (Franch and Price, 2005; Foletta et al., 2011). We found that the abundance of atrogin-1 mRNA on d +21 and that of MuRF-1 on d +3 relative to calving was affected by calving BCS; that is, they were more abundant in cows with high BCS [BCS > 3.75 and backfat thickness (BFT) > 1.4 cm] than in cows with normal BCS (BCS < 3.5 and BFT < 1.2 cm; Ghaffari et al., 2019). These data may point to upregulation of the UPS system at the mRNA level in cows with high BCS, which was accompanied by lower DMI and more pronounced negative energy balance, as well as to the differential response of the 2 ligases to the metabolic changes in early lactation as influenced by overconditioning. The mRNA abundance of MuRF-1 and atrogin-1 showed a similar pattern in control and CLA-supplemented cows with an increase from d −21 to d 1 relative to calving, followed by a decline, which was accompanied by the elevated 20S proteasome activities on d −21 and 1 (Yang et al., 2020). This probably reflects the start of protein mobilization in both groups antepartum. In the same study, atrogin-1 mRNA was 1.7-fold greater in CLA-supplemented cows than in control cows on d 21 (likely resulting from the reduced DMI and thus protein intake.
in this group), whereas that of MuRF-1 was not affected by the treatment (Yang et al., 2020). This again points to the differential response of the 2 ligases to different interventions. The differential mRNA expression of these 2 ligases in certain experimental conditions is not uncommon, as reported previously in mice (Frost et al., 2007; Yoshida et al., 2010), neonatal pigs (Suryawan and Davis, 2014), and neonatal calves (Sadri et al., 2017). Indeed, the downstream substrates of MuRF-1 and atrogin-1 are not similar, and, besides degradation, they play a role in regulating other physiological functions (Foletta et al., 2011). The myofibrillar protein components such as titin (Centner et al., 2001) and myosin light chain (MLC)1 and MLC2 (Cohen et al., 2009) have been identified as target substrates of MuRF-1, whereas the myogenic transcription factors MyoD and myogenin have been identified as targeted substrates of atrogin-1 (Foletta et al., 2011). The latter authors, in a review of published studies, proposed that MuRF-1 may be involved in the control of protein degradation and might contribute to skeletal muscle metabolism, whereas atrogin-1 may play a role in the regulation of the substrate targets affecting protein synthesis and muscle growth.

In addition to the 2 muscle-specific E3 ubiquitin ligases (i.e., MuRF-1 and atrogin-1), other components of the ubiquitin-mediated proteolytic pathway, including ubiquitin (Chibisa et al., 2008; Greenwood et al., 2009), ubiquitination of proteins (Du et al., 2005; Mann et al., 2016), ubiquitin-conjugating enzyme E2, and proteasome 26S subunit, ATPase (Chibisa et al., 2008; Greenwood et al., 2009; Ghaffari et al., 2019; Yang et al., 2020), calcium-dependent calpains (Du et al., 2004b; Chibisa et al., 2008), and calpastatin (Du et al., 2004a), as well as LC3 as a central protein in the macroautophagy pathway of protein degradation (Mann et al., 2016), have been assessed at the mRNA or protein level in the skeletal muscle of pregnant (Du et al., 2004b and 2005) and periparturient dairy cows (Chibisa et al., 2008; Greenwood et al., 2009; Mann et al., 2016; Ghaffari et al., 2019; Yang et al., 2020). In some of these studies, only mRNA abundance has been measured; however, in the case of some targets of interest, it is not known whether their activities are regulated mainly at the transcriptional level or after transcription. Therefore, further studies in which protein abundance and, more importantly, protein activity are quantified would further improve our knowledge and understanding in this area.

Feeding a nutrient-restricted diet (68.1% of NE_M and 86.7% of MP requirements) to pregnant Angus-Gelbvieh crossbred cows was found to be associated with a downregulation of calpastatin content (Du et al., 2004b) and mammalian target of rapamycin (mTOR) signaling, as well as with a higher amount of ubiquitinated proteins, pointing to a decrease in protein synthesis and accelerated protein degradation in (longissimus) muscle of nutrient-restricted cows compared with those of control animals (Du et al., 2005). Supplemental propylene glycol did not affect the mRNA abundance of calcium-dependent calpains and components of the UPS in skeletal muscle; however, the mRNA abundance for μ-calpain, ubiquitin, and ubiquitin-conjugating enzyme E2 were greater on d 15 compared with d −14 and d 38 relative to calving in dairy cows, and coincided with elevated urinary 3-MH excretion, likely reflecting increased body protein catabolism (Chibisa et al., 2008). Greenwood et al. (2009) observed a 2.1-fold greater ubiquitin mRNA abundance in skeletal muscle of dairy cows on d 3 compared with d −27 relative to calving. Probably this indicates increased proteolytic activity of this pathway. Excess energy consumption during the dry period in peripartum dairy cows did not affect the abundance of muscle myostatin and atrogin-1 mRNA, as well as immunoblot analysis of atrogin-1, MuRF-1, ubiquitinated proteins, and LC3 compared with the controlled energy group (Mann et al., 2016). However, macroautophagy in muscle tissue lysates assessed by immunoblot analysis of the ratio of LC3-II:LC3-I (as a marker of mature autophagosome formation; Stipanuk, 2009) was increased postpartum (d 4 and 21) compared with antepartum samples (d −28 and −10), regardless of the energy status prepartum (Mann et al., 2016).

### 20S Proteasome Activity

The proteasome enzyme complexes are made up of the catalytic 20S proteasome with 19S regulators (Nandi et al., 2006). The eukaryotic 20S proteasome consists of 14 α- and 14 β-subunits, arranged in a cylindrical particle as 4 heptameric rings (Tanaka, 2009). The 2 outer rings are composed of 7 distinct α-subunits each, whereas the 2 inner rings are each composed of 7 different β-subunits, 3 of which are responsible for the proteolytic activities of proteasome: 31 with caspase-like activity, 32 with trypsin-like activity, and 35 with chymotrypsin-like function (Kunjappu and Hochstrasser, 2014; Kumar Deshmukh et al., 2019). As the dysregulation of the UPS is associated with the pathogenesis of several disease states in humans (e.g., Alzheimer’s disease and cancer), the proteasome has emerged as a potential therapeutic target, and a wide range of assays have been developed to monitor proteasomal activity. Given that the catalytic signature of the proteasome may vary depending on the source, cell type, and disease state, there is a growing interest in simultaneous monitoring of all the 3 proteolytic (chymotrypsin-, trypsin-, and caspase-like) activities of the proteasome (Priestman et al., 2017).
Mobilization of skeletal muscle protein might thus be assessed by the density of the 14-kDa actin fragment in muscle biopsies taken from the periparturient cows. However, to the best of our knowledge, the performance of this marker has not been evaluated in cattle. Thus, the validity of quantifying the muscle 14-kDa actin fragment as a marker of muscle protein degradation has yet to be investigated in dairy cows under different physiological situations.

Measurement of 3-Methylhistidine and the 3-Methylhistidine:Creatinine Ratio

Assessing the breakdown rates of protein subfractions is difficult in general (Tipton et al., 2018). One approach to address the breakdown of protein subfractions is to measure 3-MH, which stems from the post-translational methylation of His residues found in the myofibrillar proteins actin and myosin (Asatoor and Armstrong, 1967; Hardy and Perry, 1969). The 3-MH is used as a marker of myofibrillar MPB because it is released into circulation during muscle degradation and cannot be further metabolized or used for protein resynthesis, but instead is excreted in the urine. Thus, urinary (e.g., Nishizawa et al., 1979; Harris and Milne, 1981; Motyl and Barej, 1986; Rathmacher et al., 1992; Plaizier et al., 2000) or circulating (e.g., Burhans et al., 1997; Doepel et al., 2002; Kokkonen et al., 2005; van der Drift et al., 2012; Ghaffari et al., 2019; Yang et al., 2020; McCabe et al., 2021) 3-MH concentrations were used as markers of whole-body myofibrillar protein breakdown in cattle.

Measuring urinary 3-MH requires several hours of total urine collection, from at least 24 h (Motyl and Barej, 1986) to several days (Plaizier et al., 2000), and the values are usually reported as daily average; thus, assuming a certain amount of 3-MH per gram of muscle, the daily rate of muscle breakdown could be quantitatively estimated using this approach. Because the content of 3-MH in muscle might change over time or with metabolic state, a biopsy sample of muscle should be taken to correct for potential differences in 3-MH content, assuming that all muscles have the same 3-MH content as the biopsied sample, which might not necessarily be true (Nissen, 1997).

Circulating creatinine concentration or urinary creatinine clearance are often used to monitor the adequacy of renal function in both animals and humans (Wyss and Kaddurah-Daouk, 2000; Braun et al., 2003). Creatinine is metabolized from creatine released by the muscles at a constant rate depending on muscle mass; thus, a decrease in muscle mass and the rate of creatinine production would be reflected by a decrease in circulating creatinine concentrations in euhydrated animals. Thus, monitoring changes in circulating creatinine concentration can be used as a marker of muscle...
mass in periparturient euhydrated dairy cows with normal renal function. Early lactation was found to be associated with a decrease in plasma creatinine concentrations in periparturient dairy cows (Bruckmaier et al., 1998a; Aeberhard et al., 2001; Megahed et al., 2019). Normalizing 3-MH to creatinine concentrations (3-MH:creatinine) is associated with smaller interindividual variations, and therefore this ratio has been recommended as a dependable measure (standardized for muscle mass) for the assessment of MPB in heterogeneous populations (Neuhäuser and Bassler, 1984). The serum ratio of 3-MH:creatinine has also been reported in periparturient dairy cows (Pires et al., 2013; Ghaffari et al., 2019; Yang et al., 2020; McCabe et al., 2021).

Although the 3-MH model of muscle proteolysis depends on several assumptions, nonetheless, in view of difficulties in assessing and estimating the rate of MPB in large animals such as dairy cows, this method can be useful and applicable for understanding the physiology of MPB regulation in periparturient dairy cows. With the urinary 3-MH model of measuring MPB, if combined with additional measurements of both urinary creatinine and 3-MH content of the muscle, a daily rate of muscle breakdown could be quantitatively estimated, and short-term insight into nutritional or physiological effects on muscle proteolysis could potentially be achieved.

Measurement of Skeletal Muscle Thickness: Ultrasonography

Ultrasonography has already been widely used in the beef industry as a selection and evaluation tool (Gillis et al., 1973). Ultrasonographic measurement of subcutaneous BFT has also been used over the last 20 yr in dairy cows to determine body fat reserves, to test the accuracy of visual body condition estimates, and to evaluate the rate and extent of fat mobilization (Domcq et al., 1995; Schröder and Staufenbiel, 2006; Bell et al., 2018). In some studies, using a combined approach, ultrasound BFT measurements were performed in combination with longissimus dorsi muscle thickness (Schwager-Suter et al., 2000). This approach seems more effective and appropriate for assessing body condition, as it quantifies the simultaneous contribution of both fat and muscle reserves to BCS, as well as for monitoring the rate and extent of muscle mobilization in periparturient dairy cows (Bruckmaier et al., 1998a; Jaurena et al., 2005; Kokkonen et al., 2005; van der Drift et al., 2012; Megahed et al., 2019; McCabe et al., 2021; Siachos et al., 2021).

The thickness of the longissimus dorsi muscle is measured at the levels of loin and thoracic area, but the former area seems to be preferred, with possible improved resolution in measuring muscle thickness in this area due to lack of interference by overlying ribs (Bruckmaier et al., 1998b; Jaurena et al., 2005; van der Drift et al., 2012; Megahed et al., 2019). Several works have also already used ultrasonography and image analysis to assess intramuscular fat content in beef cows (Yang et al., 2006; Irie and Kohira, 2012; Nunes et al., 2015; Giaretta et al., 2018). Megahed et al. (2019) have also estimated changes in intramuscular fat of the gluteus medius and longissimus dorsi muscles in periparturient dairy cows by quantifying muscle echogenicity through grayscale histogram analysis. They found that periparturient decreases in muscle thickness reflect mobilization of both skeletal muscle and intramuscular fat (Megahed et al., 2019). As already acknowledged (Young et al., 2015; Megahed et al., 2019), the use of mean grayscale analysis as an index of intramuscular fat has its own limitations, as echogenicity units cannot be directly quantified in terms of intramuscular fat percentage. Taken together, repeated ultrasound measurements of longissimus dorsi muscle thickness have the potential to provide a useful on-farm method to monitor changes in muscle depth over time and between nutritional treatments, but do not allow quantification of MPB.

D2O Dilution Method

This method is a quantitative technique used to estimate body composition in humans and domestic species. Deuterium is a stable and nonradioactive isotope of hydrogen, and this technique can potentially be used to assess longitudinal changes in body composition during the periparturient period as well as before and after an intervention. However, this approach does not apply specifically to muscle protein. Two studies have previously used the D2O dilution technique to estimate body composition in dairy cows supplemented with fat or protein at −2, 5, and 12 wk relative to calving (Komaragiri and Erdman, 1997; Komaragiri et al., 1998). The infusion of D2O into the jugular vein at the dosage rate of 150 (Komaragiri and Erdman, 1997) or 250 mg/kg of BW (Komaragiri et al., 1998) combined with multiple blood sampling to estimate empty and total body water, and subsequently empty body protein and empty body fat, were calculated using the equations described by Andrew et al. (1994). Estimates of body fat and body protein mobilization were, respectively, a mean of 54 and 21 kg by wk 5 postpartum regardless of dietary protein level (16 or 19% CP) in dairy cows; although cows continued to mobilize 18 kg of body fat between 5 and 12 wk postpartum, no change in the amount of empty body protein occurred during this period (Komaragiri and Erdman, 1997). In a companion study using a similar technique, Komaragiri et al.
oral bolus of D2O, merits further research in dairy cows. Sampling media, such as urine and saliva, following an oral bolus of D2O followed by a single (a time when isotopic enrichment is reached within the body fluid) or multiple post-dose sampling also from other matrices than blood, including urine, saliva, milk, and exhaled air (Smith et al., 2002; Colley et al., 2007). The applicability and accuracy of this method when using other sampling media, such as urine and saliva, following an oral bolus of D2O, merits further research in dairy cows.

Limitations Associated with Indirect Measures of Muscle Protein Breakdown

An important limitation of the indirect measurements of MPB is that they provide markers rather than quantitative values of MPB. A weakness of assessing changes of specific components related to MPB pathways at the mRNA level is that such changes may not always reflect physiological changes in muscle metabolism or mass (Atherton et al., 2016). All components of the MPB pathways are involved in various steps, regulating the pathways, but their relative importance in different regulatory steps may differ, and the response of mRNA expression of multiple genes may be variable (Nedergaard et al., 2007; Reitelseder et al., 2014). Moreover, changes in mRNA expression of components of the MPB pathways seem to depend on the time of assessment and the type of muscle under study (Atherton et al., 2016). Due to the fiber composition, a wide range of functional and metabolic variations in muscle tissue have been found (Gunawan et al., 2007). Inconsistent changes in mRNA abundance of components of the ubiquitinin-dependent system were reported in periparturient (Chibisa et al., 2008; Ghaiffari et al., 2019; Yang et al., 2020) and in acidotic dairy cows (Mutsvangwa et al., 2004). Such variable responses may suggest different functional properties of the proteins, making the interpretation of these results challenging. Thus, these limitations should be carefully taken into account when results using these methods are appraised, to avoid overinterpretation of the data. The changes in mRNA abundance can be assessed along with other parameters (e.g., enzyme activity) in the same muscle tissue sample.

Another important issue is proper choice of stable reference genes (a minimum of 2 or 3) to be used to normalize the raw reverse-transcription quantitative real-time PCR (RT-qPCR) data of a given gene of interest, which is often performed without critical evaluation (Chapman and Waldenström, 2015). The improper normalization of mRNA data can have a profound effect on the conclusions drawn from studies of gene expression. The guidelines for adequate reporting of RT-qPCR studies have been published (MIQE guidelines; Bustin et al., 2009), and the number of studies specifically testing the stability of reference genes are increasing over time; nonetheless, uncritical use of single and unvalidated reference genes to normalize RT-qPCR data is still widespread. A concern about measuring urinary or circulating 3-MH is that they do not solely reflect the specific breakdown of myofibrillar protein, as 3-MH is also released from tissues other than skeletal muscle, such as cardiac and smooth muscle, and might also be affected by renal function or blood flow. Furthermore, a specific concern about assessing 3-MH in periparturient dairy cows is that a potential difference measured between precalving and postcalving period values could also be partly due to uterine involution. Dry weight of the uterus, consisting predominantly of myofibrillar protein, has been estimated to increase on average from 250 to 2,600 g during pregnancy in cattle (Kaidi et al., 1995), which takes an average of 45 d for uterine involution to be completed in cows (Jainudeen and Hafez, 1993). However, the contribution from postpartum uterine involution to the overall rate of protein catabolism is estimated (by 3-MH excretion) to be small (Plaizier et al., 2000) compared with the intensity of skeletal muscle metabolism associated with negative nitrogen balance resulting from insufficient nutrient intake to meet nutrient requirements in periparturient cows (Motyl and Barej, 1986; Maltz and Silanikove, 1996; Komaragiri and Erdman, 1997; Komaragiri et al., 1998). Moreover, the amount of 3-MH in tissues other than skeletal muscle in growing Holstein cattle has been demonstrated to be relatively small (about 6.6%; Nishizawa et al., 1979).

Another important issue concerned with the urinary 3-MH model is that all 3-MH produced from the muscle is derived from endogenous muscle proteolysis and not from exogenous sources (i.e., dietary 3-MH). The presence of 3-MH in cattle feed has already been shown (cocksfoot hay and mixed concentrate contained 2.40 and 7.73 mg/kg, respectively; Nishizawa et al., 1979). Assuming that absorbed dietary 3-MH is quantitatively excreted in the urine, the contribution of 3-MH in feedstuffs was calculated to be approximately 30%
of the urinary 3-MH (Nishizawa et al., 1979). Thus, measuring 3-MH content in feed might help in better estimating the catabolic rates of skeletal muscle protein from urinary excretion of 3-MH.

Some limitations should be considered when performing and interpreting muscle measurements derived from ultrasound, such as the inability to account for intramuscular fat (located among and between skeletal muscle fibers) that might increase the ultrasonographically assessed diameter. It is thus possible that the ultrasonographically determined decrease in muscle thickness during the periparturient period (Bruckmaier et al., 1998a; van der Drift et al., 2012; Megahed et al., 2019) is partly related to a decrease in intramuscular fat (located among and between skeletal muscle fibers) that might increase the ultrasonographically assessed diameter. It is thus possible that the ultrasonographically determined decrease in muscle thickness during the periparturient period (Bruckmaier et al., 1998a; van der Drift et al., 2012; Megahed et al., 2019) is partly related to a decrease in intramuscular fat and not solely skeletal muscle tissue.

An important limitation associated with the D2O dilution technique is that this method is used to estimate body composition and does not apply specifically to muscle protein. In this technique, empty body water estimation did not include water associated with gastrointestinal contents or water in the conceptus. Thus, it is not clear to what extent the estimated values using this technique might be influenced by changes in gut fill or body condition, and whether monitoring rapid changes over short periods is possible. In addition, multiple blood sampling, analysis, and costs associated with the technique seem to have limited the use of this technique as a practical and welcome method to estimate body composition and to (indirectly) monitor body protein breakdown in periparturient dairy cows.

**PROTEOLYSIS-DEPENDENT REGULATION OF PROTEIN SYNTHESIS**

Muscle protein turnover is the continual process reflecting the balance between rates of protein synthesis and breakdown. Protein synthesis and degradation are intimately connected and tightly controlled to ensure proper protein homeostasis. In fact, MPB systems are often regulated by complex mechanisms that impinge on both protein synthesis and degradation, so that the increase in protein synthesis and the suppression of proteolysis are linked events (Sandri, 2013). However, in certain situations, protein synthesis might increase during muscle atrophy (Sandri, 2013; Sartori et al., 2021). This is because a portion of the AA released by MPB directly stimulates protein synthesis through mTOR (Liu and Sabatini, 2020), and blocking this mechanism leads to exacerbated muscle loss (Quy et al., 2013).

The mTOR signaling pathway plays a central role in regulating cell growth and metabolism in eukaryotes (Wullschleger et al., 2006). The core kinase mTOR is present in 2 functionally and structurally distinct multiprotein complexes, mTORC1 and mTORC2 (Sarbassov et al., 2005; Inoki and Guan, 2006; Yang et al., 2008). The mTORC1 is a nutrient and energy sensor, in particular of AA (Hay and Sonenberg, 2004). Among AA, Leu is regarded as the most effective activator of mTORC1 (Dodd and Tee, 2012). The mTORC2 responds to growth factors (e.g., insulin and IGF-1) but is largely nutrient-insensitive. A reciprocal relationship between mTOR and lysosomal-dependent protein degradation has been shown, in which AA released during lysosomal proteolysis modulate mTOR activity (Sancak et al., 2010; Narita and Inoki, 2012; Condon and Sabatini, 2019).

Indeed, the Ragulator-Rag complex, which senses lysosomal AA, promotes the localization of mTORC1 at the lysosomal surface. The formation of an mTOR–autophagy spatial coupling compartment is necessary for AA-mediated activation of mTOR and autophagy in a mutually reinforcing manner. This feedback mechanism, proposing simultaneous activation of anabolic and catabolic processes, is thought to be a critical mechanism for cell survival under conditions of nutrient depletion, and this also likely allows cells to translate essential proteins that are transcribed at the onset of starvation (Condon and Sabatini, 2019; Lawrence and Zoncu, 2019). Such a mechanism might be relevant in periparturient dairy cows, as the increased mRNA abundance of muscle-specific ligases (MuRF-1 and atrogin-1) coincides with greater mRNA abundance of key components of mTOR signaling (Ghaffari et al., 2019; Yang et al., 2020). This is likely a feedback mechanism that prevents excessive MPB during the periparturient period in these cows. However, it remains to be elucidated to what extent the observed regulation of mTOR and UPS at the transcriptional level may correlate with protein synthesis rates in periparturient dairy cows.

**CONCLUSIONS AND FUTURE RESEARCH DIRECTIONS**

Our understanding of the regulation and response of MPB to the physiological and metabolic changes and nutrition in periparturient dairy cows is still incomplete, which is at least partly due to the technical difficulties of measuring MPB in vivo. The AV balance methods, with the use of either a 2- or a 3-compartment model measure protein breakdown in absolute units rather than relative units; however, they are relatively invasive and do not seem to be applicable in studies with periparturient dairy cows. The various tracer methods that rely on the precursor-product principle to measure FBR are classified by whether they need an isotopic steady state or multiple tracers and by how many biopsies should be obtained. The pulse tracer injection method...
is worth considering in future studies in periparturient dairy cows as a less invasive approach that does not require measurement at isotopic steady state (if the arterial and intracellular enrichments are measured at 3 time points) and allows measuring both muscle protein FBR and FSR within a short time frame. Some new methods have been developed in humans for assessing MPB in various situations, but, to date, they have not been validated in periparturient dairy cows. Simultaneous and repeated measurements of MPB using static markers of MPB pathways, metabolic indicators (3-MH and 3-MH:creatinine), proteolytic enzyme activities, and changes in muscle thickness seem to be applicable methods and can thus provide important data to enhance our understanding of the timing and contribution of the various components of the MPB machinery to MPB through the periparturient period. However, indirect measurements of MPB provide markers rather than quantitative values of MPB. It should be noted that the individual components of the 3 main systems responsible for driving MPB are interconnected and do not work separately. Thus, changes in one isolated component might or might not be responsible for triggering a change in overall MPB. Applicability and accuracy of bolus ingestion of D2O combined with urine, saliva, or milk sampling merit remain to be evaluated in future body composition research in dairy cows. Limited data are available about how various nutrition interventions during the periparturient period influence the degradation of particular proteins in muscle. Thus, in addition to determining the genetic basis for the extent and timing of peripartum MPB, future research should also focus on the potential interactive role of nutritional modifications with muscle metabolism and their links with performance, reproduction, health, and immune status in periparturient dairy cows.

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