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Fertility and the transition dairy cow

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Abstract. The transition from pregnancy to lactation (i.e. the transition period) is a time of significant metabolic challenge, with a several-fold increase in a cow's requirement for energy, protein and minerals within days of calving. A successful transition involves the initiation and coordination of changes in multiple tissues that facilitate the provision of these nutrients to the cow and, more specifically, to the mammary gland, often at the considerable expense of other tissues. Failure to coordinate the necessary changes effectively results in transition period maladaptation, which can broadly be grouped into three categories: (1) negative energy balance and metabolic diseases associated with energy metabolism; (2) immune dysfunction and inflammation; and (3) metabolic diseases associated with mineral deficiency. Because reinitiation of ovarian activity, follicle recruitment, ovulation, fertilisation and, potentially, even maternal recognition of pregnancy and implantation occur against the backdrop of this metabolic and immunological disturbance in early lactation, the role of nutrition in ensuring a smooth transition between the pregnant and lactating state is important. In this paper we integrate recent research findings with previous knowledge of the interaction between transition cow metabolism and nutrition and reproductive outcomes, and offer new insights into key elements of successful cow management to avoid transition 'maladaptation' and improve pregnancy rates.

Additional keywords: energy balance, exosomes, immunity, inflammation, metabolic disease, NSAIDs.

Introduction

Coincident with increasing milk production through genetic and husbandry advances, reproductive efficiency in dairy cows has been in decline for more than 20 years (Roche *et al.* 2000, 2011; Royal *et al.* 2000): cows take longer to return to oestrus, exhibit poorer signs of oestrus, may have poorer conception rates and have greater embryo loss (Lucy 2001; Roche *et al.* 2011). The reasons underlying the negative association between milk production and fertility are complex and multifactorial, but physiological changes to support the supply of nutrients to the mammary gland, at the expense of other tissues, are implicated (Lucy 2001; Chagas *et al.* 2009; Lucy *et al.* 2009; Friggens *et al.* 2010). This is consistent with the negative genetic correlation between milk production and health (Uribe *et al.* 1995; Pryce *et al.* 1997; Ingvartsen *et al.* 2003) and milk production and reproduction (Berry *et al.* 2003). However, the negative association is not universal (Bello *et al.* 2012). For example, Cummins *et al.* (2012*a*, 2012*b*, 2012*c*) reported divergent fertility phenotypes with similar milk production, and there is evidence of fertility improving following the inclusion of a fertility subindex in multiple countries' national breeding objectives (Berry *et al.* 2014).

For dairy cows, high reproductive efficiency requires a successful pregnancy within 3 (seasonal production system) to 4 (non-seasonal production system) months after calving (De Vries 2006; Roche *et al.* 2000). Achieving this requires the quick resolution of a range of physiological, endocrine and tissue remodelling processes during a particularly nutrient-demanding and physiologically and immunologically challenging period.

*John Roche was the invited contributor. Other authors are listed alphabetically.

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Therefore, failure to 'transition' effectively between the pregnant and lactating state has implications for reproductive success.

The transition period: adapting to the onset of lactation

During the 6- to 8-week period between late pregnancy and early lactation, commonly referred to as the 'transition period', significant metabolic adaptations occur in all mammals; however, intensive selection for milk production in the modern dairy cow has resulted in even more extreme physiological changes to ensure nutrient supply to the mammary gland is prioritised above other tissues. For example, at 4 days after calving, a cow producing 30 kg milk requires 2.7-, 4.5- and 2.0-fold the amount of glucose, fatty acids (FAs) and amino acids, respectively, than she did 4 days before calving, and that is just for milk production (Bell 1995). In fact, because of the metabolic requirements of the gland, particularly in early lactation, it has been suggested that the cow should be considered an appendage to the mammary gland, rather than vice versa (Bauman *et al.* 2006). Nutrient requirements of non-mammary tissues also undergoing peripartal hypertrophy to supply the mammary gland with nutrients (e.g. liver, intestine) add to the metabolic challenge.

Fulfilling the abrupt increase in nutrient requirements at the onset of lactation requires major changes to multiple tissues in a very short time. Mobilisation of body fat is extreme for 10–15 days after calving (Fig. 1); this is achieved by a combination of increased lipolysis and decreased rates of lipogenesis and FA re-esterification in adipose tissue and the net release of FA and glycerol into the bloodstream (Bell 1995; Drackley 1999; Drackley *et al.* 2001; Roche *et al.* 2009). The homeorhetic changes required to achieve this tissue mobilisation appear to be primarily under genetic control (McNamara and Hillers 1986; McNamara 1991), with nutrition during the first 5 weeks of lactation having very little effect (McCarthy *et al.* 2007; Roche 2007; Roche *et al.* 2006, 2009). Plasma FAs are efficiently used for mammary triacylglycerol (TAG) synthesis and can be oxidised in most non-mammary tissues. In addition, glycerol is a readily assimilated precursor for hepatic gluconeogenesis (Roche *et al.* 2013*a*).

Mechanisms of a more moderate mobilisation of amino acids from so-called 'labile tissue protein reserves' are less well understood. However, evidence points to suppression of protein synthesis and possibly increased proteolysis in skeletal muscle (Bell *et al.* 2000). There is speculation that there may be some diversion of amino acids from splanchnic tissue synthesis in the immediate post-partum period and the secretion of export proteins to support much of the suddenly increased hepatic requirement for glucogenic substrate for at least a few days after calving (Bell *et al.* 2000; Roche *et al.* 2013*a*). However, recent studies of substrates used for hepatic gluconeogenesis in the early post-partum period do not support this speculation (Larsen and Kristensen 2012).

The onset of lactation places such a large demand on mechanisms involved in maintaining calcium homeostasis that most cows develop some degree of hypocalcaemia at calving. In some cases, plasma calcium concentrations become too low to support nerve and muscle function, resulting in parturient paresis or milk fever (Goff and Horst 1997*a*). Adaptations to increase the blood supply of calcium very soon after calving include increased intestinal active transport, increased resorption of bone stores and decreased urinary excretion of calcium (Horst *et al.* 2005).

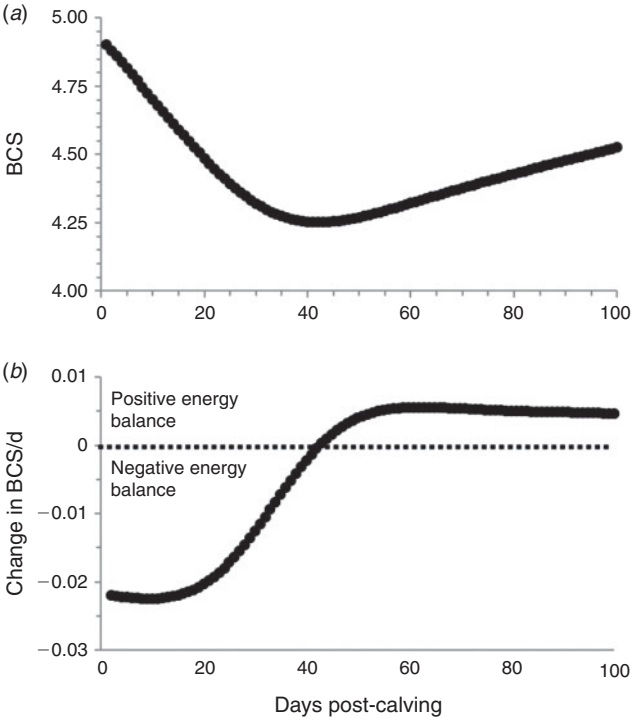

Fig. 1. (*a*) Early lactation body condition score (BCS) profile and (*b*) the daily rate of change in BCS. Cows are in negative energy balance for 42 days after calving, but the rate of BCS loss is very high for the first 10–20 days and declines rapidly thereafter. BCS was measured using a 10-point scale, where 1 is emaciated and 10 is obese (Roche *et al.* 2004). Adapted from Roche *et al.* (2007*b*).

The concept of homeorhesis (Bauman and Currie 1980) implies the agency of endocrine or neuroendocrine factors capable of responding to physiological and environmental changes and of simultaneously affecting disparate metabolic functions in multiple tissues. Roche *et al.* (2013*a*) provide a full review of the homeorhetic and homeostatic regulation of physiological changes associated with the transition from pregnancy to lactation. Briefly, the transition dairy cow uncouples the somatotropic axis (Lucy *et al.* 2009; Grala *et al.* 2011), has lower insulin production and greater insulin resistance (Lucy 2001; Chagas *et al.* 2009) and is in an increased state of subacute or chronic inflammation (Bradford *et al.* 2015). All these conditions are, to some degree, interdependent and occur to ensure that glucose, in particular, is partitioned to the mammary gland at the expense of other tissues (e.g. muscle, immune cells). Phenotypically, these metabolic changes result in a greater degree and duration of body condition loss in early lactation and, as a result, a greater risk of metabolic diseases associated with excessive loss of condition (e.g. ketosis, fatty liver).

As well as affecting metabolic health, the transition period is also the period of greatest risk for infectious diseases, with the risk of mastitis and metritis greatly increased in the weeks

immediately after calving; these diseases have implications for reproduction (LeBlanc 2008). Despite this, almost as if to scuttle the proverbial 'reproduction ship' before it sets sail, the functionality of the innate immune system is diminished during the weeks immediately before and after calving, with a well-reported reduction in the number and effectiveness of immune cells in blood (Goff and Horst 1997*a*; Heiser *et al.* 2015; Crookenden *et al.* 2016*a*). There is also a relationship between metabolic state and peripartum immune function (Contreras and Sordillo 2011; Sordillo and Raphael 2013; Bradford *et al.* 2015; Crookenden *et al.* 2016*b*, 2017; Sordillo 2016), wherein the functioning of the immune system of cows in metabolic distress is further reduced.

In summary, the diverse and yet orchestrated nature of the metabolic adaptations to support lactation involves many tissues (i.e. homeorhesis; see Bauman and Currie 1980). If any of the adaptation processes are compromised, this can result in a cascade of metabolic problems, reduce the functionality of the immune system, exacerbate the controlled peripartum inflammation, increase the risk of metabolic and infectious diseases and even result in death. The purpose of this review is to integrate recent research findings with previous knowledge of the interaction between transition cow metabolism and nutrition and reproductive outcomes, and to offer some new insights into key elements of successful management of the cow to avoid transition 'maladaptation'.

Physiological processes underpinning fertility and subfertility as they relate to the transition period

The chain of reproduction events between when a cow calves and recalves is long and complex, but can, for all intents and purposes, be split in two when considering the transition period (see Roche *et al.* 2011): (1) preovulatory reproductive failure, with transition cow metabolic state and nutrition potentially affecting the timing of return to oestrus; and (2) postovulatory reproductive failure, when the transition cow metabolic state and nutrition may affect oocyte quality, fertilisation, embryo survival and implantation.

Preovulatory reproductive failure

Preovulatory reproductive failure is primarily a function of the timing of return to oestrus post partum. An early resumption of oestrous cycles following calving is important, because delays result in reduced conception and pregnancy rates. Thatcher and Wilcox (1973) reported a quadratic decline in services per conception with increasing numbers of recorded oestrous cycles before 60 days in milk. Recently, a greater failure of embryos to reach the 7 day blastocyst stage if not previously detected in oestrus was identified compared with contemporaries on a second or later oestrous cycle (D. Berg, unpubl. data). Therefore, the effects of metabolic changes during the transition between pregnancy and lactation on the processes underpinning preovulatory reproductive failure must be understood (Santos *et al.* 2016).

Postovulatory reproductive failure

Postovulatory reproductive failure is a major component of poor reproductive performance in dairy cows, but again can potentially be divided into two categories: (1) maternal–embryo interaction; and (2) early blastocyst stage failure.

Historically, reproductive failure was accepted as a failure at the level of maternal–embryo communication. Fertilisation rates of approximately 90% were expected (Diskin 2008) but, based on an average calving rate of 55%, Sreenan and Diskin (1986) calculated an embryonic and fetal mortality rate (excluding fertilisation failure) of approximately 40% for 'fertile' cows and 55% for 'subfertile' cows. They estimated that 70–80% of the loss occurred between Days 8 and 16 after insemination. These early findings have been substantiated (Walker *et al.* 2012; Wiltbank *et al.* 2016). Using transcriptomic analyses of endometrium harvested from fertile and subfertile dairy cow strains, Walker *et al.* (2012) provided support for this proposed timing of embryo loss, at least in the North American Holstein–Friesian breed. The collective results indicate that reproductive failure was primarily a result of a failure at the maternal recognition and implantation stage, implicating the uterine environment in reproductive failure (Walker *et al.* 2012). These changes may relate to differences in progesterone profiles between the different dairy cow strains investigated (Meier *et al.* 2009), because progesterone is a primary driver of endometrial gene expression and conceptus elongation (Forde *et al.* 2009; Spencer *et al.* 2016), and could be the result of either inadequate production or increased hepatic metabolism.

More recently however, our team identified that the critical period for early embryo loss in the fertile strain mentioned previously (i.e. moderate-producing cows in New Zealand) was in the first week after fertilisation, with approximately 66% of the embryos ultimately lost, non-viable by Day 7 (D. Berg, unpubl. obs.). These results implicate factors associated with oocyte quality, follicle development and possibly oviducal environment in fertilisation failure (Sartori *et al.* 2010).

Differences in the timing of embryo loss point to different reasons for early embryo loss, which is consistent with the inferior follicular development, progesterone production and uterine environment reported by Cummins *et al.* (2012*b*) in a subfertile strain of dairy cow. Nevertheless, considering that the timing of reinitiation of follicular waves post partum, the recruitment of the dominant follicle and the development of the oocyte ultimately fertilised, as well as uterine involution and the resolution of any infection or inflammation, all occur during the transition period for many cows, it is reasonable to assume that any perturbation to dairy cow metabolic or immunological state will increase the risk of postovulatory reproductive failure (Leroy *et al.* 2005, 2008; Lonergan 2011). The contribution of features of transition cow maladaptation to reproduction and best practice management guidelines to improve the likelihood of postovulatory reproductive success are discussed below.

'Maladaptation' during the transition period and its role in reproductive failure

The transition period, although short, is when most metabolic and infectious diseases occur during the dairy production cycle (Roche *et al.* 2013*a*). These diseases are a result of the cow's physiological processes failing to adapt to increased nutritional requirements for lactation and is referred to as transition period 'maladaptation'.

Metabolic diseases are complex disorders that occur when the cow's ability to adjust to a major physiological change (e.g. pregnancy to lactation) is compromised. They have been a persistent problem for farmers for centuries, with milk fever, for example, first documented in 1793 in Germany (Schultz 1971) and ketosis reported in the US as early as 1849 (Udall 1943). Most metabolic disorders stem from nutritional inadequacy or a failure to prime metabolic processes for the change from pregnancy demands to lactational demands. In comparison, infectious diseases generally reflect an inability of the cow's immune system to withstand a bacterial or viral challenge. A focus of any transition cow management program must be to ensure that peripartum disease (i.e. both clinical and subclinical) is minimised, thereby maximising the likelihood of an early return to oestrus and a successful pregnancy outcome.

The orchestrated changes to cow physiology during the transition period are complex and provide several 'opportunities' for 'maladaptation' (Thatcher *et al.* 2011). Nevertheless, they can be grouped into three broad subject areas, which will allow us to directly discuss their interaction with reproduction: (1) negative energy balance (NEB) and metabolic diseases associated with energy metabolism; (2) immune dysfunction and inflammation; and (3) metabolic diseases associated with mineral deficiency.

Early lactation NEB and metabolic diseases associated with energy metabolism

Because of the asynchronous increase in energy demands relative to supply after calving, most cows mobilise a large amount of adipose tissue during the first 2–4 months of lactation (Roche *et al.* 2009) and most especially during the first 10–20 days after calving (Fig. 1; Roche *et al.* 2007*b*). This results in high FA concentrations circulating in the blood during early lactation. High blood FAs can lead to metabolic diseases associated with a failure to fully oxidise FAs, most notably ketosis and hepatic lipidosis.

Ketone bodies are intermediaries in the breakdown of FAs and can accumulate in the blood when large amounts of body fat are mobilised and there is insufficient carbohydrate to facilitate β-oxidation. The clinical state of this disease is referred to as ketosis and is defined by blood concentrations of β-hydroxybutyrate (BHB) >2 mM, with hyperketonaemia or 'subclinical ketosis' defined as blood BHB concentrations between >1.2 and 1.4 mM (Duffield *et al.* 2009; Gordon *et al.* 2013).

There are three types of ketosis. Type I ketosis is a result of an unexpected drop in dry matter (DM) intake, particularly in high-producing cows (spontaneous ketosis). This type of ketosis can be prevented by ensuring cows are adequately fed or by ensuring any feed restriction is imposed gradually (e.g. over a week). Reducing milking frequency may also reduce the risk of Type I ketosis (Kay *et al.* 2013).

Type II ketosis occurs to a degree in all cows 3–4 weeks after calving, but the clinical condition generally occurs in over-conditioned cows and can occur during the first 10 days after calving: the risk of Type II ketosis doubles when calving body condition score (BCS; a measure of fatness, with low values indicating emaciation and higher scores indicating obesity) increases from 5.5 to 6.0 over a 10-point scale (Gillund *et al.* 2001). Prevention should be through feeding management in late lactation and during the non-lactating period before calving (Roche *et al.* 2015). Mature cows should be fed to achieve a calving BCS of 5.0 (10-point scale) at calving (Roche *et al.* 2009).

The third type of ketosis is silage ketosis. In addition to the two main types of ketosis, cows can also get ketosis from consuming poor-quality silage. Silage that has undergone a secondary fermentation will increase blood BHB concentrations and the risk of ketosis. Such silage should not be fed to transition dairy cows.

Hepatic lipidosis is a metabolic disorder that occurs when the hepatic uptake of FAs exceeds their oxidation and secretion by the liver. When this happens, fat is re-esterified to TAG and released into the circulation as very low-density lipoprotein. During periods of NEB, hepatic capacity for FA re-esterification increases in the dairy cow. However, the export rate from the liver remains low; as a result, TAG accumulates within the hepatocyte. This is commonly referred to as fatty liver disease (Bobe *et al.* 2004).

Effects on reproductive function

Although the literature is consistent that dairy cow reproductive function is compromised as the severity of NEB increases (Roche *et al.* 2009) and when BHB (Raboisson *et al.* 2014; Shin *et al.* 2015) and TAG (Bobe *et al.* 2004) thresholds are exceeded, it is often not possible to separate the effects of blood FA concentration from those of blood BHB and liver TAG concentrations. Therefore, we discuss the effect of NEB as a general state that is likely to include high blood FA and BHB concentrations and high liver TAG concentrations, but where appropriate will refer to the actual metabolic diseases.

The timing of BCS loss (NEB), high blood FA and ketone body concentrations and high liver TAG concentrations coincide with the reinitiation of ovarian activity, the development of the follicles that will provide oocytes for fertilisation, the involution and remodelling of the uterine and oviducal structures and, for many cows (particularly those in seasonal calving systems where the aim is to rebreed within 80 days of parturition), fertilisation, blastocyst and even post-hatching conceptus growth and development.

Because of the coincidental occurrence of these processes and metabolic states, NEB, BHB and liver TAG can affect both pre- and postovulatory reproductive function (Villa-Godoy *et al.* 1990; Mackey *et al.* 2000; Diskin *et al.* 2003; Pushpakumara *et al.* 2003; Chapinal *et al.* 2012; Shin *et al.* 2015; Lüttgenau *et al.* 2016). For example, early lactation energy balance is negatively associated with the duration of the post-partum anoestrous interval (PPAI), with longer durations to first ovulation associated with increasingly severe NEB (Diskin *et al.* 2003; Patton *et al.* 2007; Roche *et al.* 2007*a*; Garnsworthy *et al.* 2008), and positively associated with pregnancy rate to first and subsequent inseminations (Diskin *et al.* 2003; Roche *et al.* 2009; Walsh *et al.* 2011). Similarly, calving to first service interval increased and the odds of a successful pregnancy to first service

diminished with blood BHB concentrations >1.4 mM and FA concentrations >1.0 mM in early lactation (Raboisson *et al.* 2014).

There are many reasons for the negative effects of NEB and metabolite concentrations reflective of maladaptation to lactation on reproductive function. For example, follicle growth and size is reduced during NEB and follicles emerging after the NEB nadir, rather than before, have greater growth and diameter, enhanced oestradiol production and are more likely to ovulate (Beam and Butler 1999; Diskin *et al.* 2003). In comparison, the dominant follicle in cows in NEB had to be larger to establish blood oestradiol concentrations capable of triggering ovulation, which was therefore delayed (Beam 1995). Because oocytes from large and aged follicles are less fertile, this is, at least in part, the reason NEB reduces subsequent conception rates, even if the animal has returned to positive energy balance. Physiologically, NEB manifests itself in delayed ovarian activity by impinging on the pulsatile secretion of LH, reducing follicular responsiveness to LH and FSH and ultimately by suppressing follicular oestradiol production (Diskin *et al.* 2003).

The physiological mechanisms by which NEB may affect follicle development are not completely understood, but are postulated to involve insulin-like growth factor (IGF) 1. IGF1 has been reported to alter follicle sensitivity to gonadotrophins (Garnsworthy *et al.* 2008) and oocyte quality (Lucy 2001), and has been implicated in uterine receptivity and embryo implantation (Robinson *et al.* 2000). NEB in early lactation has also been reported to alter the IGF system in the oviduct and endometrium (Fenwick *et al.* 2008; Wathes *et al.* 2009), potentially affecting uterine receptivity and embryo implantation. Robinson *et al.* (2000) reported that components of the IGF system were differentially expressed in the uterus and that factors that affect the expression of this system may alter uterine function through modulation of uterine glandular activity and the development of uterine caruncles. From the collated data, it is reasonable to conclude that factors that result in disturbances within the IGF system are detrimental to follicle and embryo development, and perhaps provide a mechanism for reduced fertility in these animals (Roche *et al.* 2011).

In addition to the effects of energy balance on follicle development, time to first ovulation and oocyte or embryo quality, NEB in early lactation can alter the necessary postpartum uterine inflammatory response (Bromfield *et al.* 2015; Bicalho *et al.* 2017; Sheldon *et al.* 2017*a*, 2017*b*), thereby delaying uterine repair (Wathes *et al.* 2009), reducing innate immune response (van Knegsel *et al.* 2007) and delaying the elimination of bacteria from the uterus (Lewis 1997), increasing the risk of metritis and endometritis (Duffield *et al.* 2009; Compton *et al.* 2015).

In summary, early lactation NEB has been implicated in both pre- and postovulatory reproductive failure because a more severe NEB increases the duration of PPAI, reduces the quality of the ensuing oocyte and delays the full involution and resolution of the post partum uterus. This manifests itself in an extended PPAI and lower submission and 6- and 12-week pregnancy rates. Therefore, an aim of transition cow management must be to minimise the extent and duration of early lactation NEB.

Managing early lactation NEB to prevent metabolic diseases associated with energy metabolism and improve reproductive function

The aetiology of metabolic diseases associated with energy metabolism is complex and multifactorial, but central to their occurrence is the excessive mobilisation of adipose reserves before calving and during early lactation, and a failure of the cow to rapidly increase intake after calving (DM intake (DMI)). Thresholds for blood FA concentrations before (0.3–0.4 mM) and after (0.7–1.0 mM) calving and BHB concentrations after calving (1.2–1.4 mM) have been reported that, when exceeded, reduce fertility (McArt *et al.* 2013; Raboisson *et al.* 2014). Two factors are particularly important in ensuring these thresholds are not breached: (1) calving BCS; and (2) precalving feeding level.

The positive effect of calving BCS on fertility outcomes has been well established for decades, with a linear decline in PPAI and a linear increase in pregnancy rate reported with increasing calving BCS and BCS at the time of insemination (Buckley *et al.* 2003; Roche *et al.* 2009). Calving BCS does not affect precalving blood FA concentrations (Roche *et al.* 2013*b*, 2015), but post-calving BCS loss, blood FA and BHB concentrations and liver TAG all increase with increasing calving BCS (Akbar *et al.* 2015; Roche *et al.* 2013*b*). For example, Roche *et al.* (2013*b*) reported that 40% of cows that calved at BCS 5.5 (10-point scale; Roche *et al.* 2004) were hyperketonaemic and mean herd blood FA concentrations were 0.93 mM after calving, compared with 0% of cows and 0.71 mM blood FA concentrations for cows that calved at BCS 4.5. Therefore, managing BCS in late lactation and during the non-lactating period to ensure that cows are not overconditioned at calving (i.e. calve at BCS 5.0 on a 10-point scale; ~3.0 on a five-point scale; Roche *et al.* 2004) is a key factor in balancing the risk of post-calving metabolic diseases and maximising the odds of a successful reproduction event (Dann *et al.* 2006; Roche *et al.* 2017).

Although highlighting that post-partum NEB (i.e. high blood FAs) and the inability to completely oxidise FAs (i.e. high blood BHB) are important to reproductive function, the threshold for blood FA concentrations before calving reported by Raboisson *et al.* (2014) highlighted that NEB before calving also negatively affects reproductive function. This is consistent with the positive relationship between prepartum blood FA and BHB concentrations and the incidence of metabolic diseases post partum (Dyk 1995; Sheehy *et al.* 2017), and supports the long-held premise that DMI must be maximised before calving (Boutflour 1928; Hutton and Parker 1973; Bertics *et al.* 1992; Grummer 1995). However, such a recommendation is inconsistent with more recent research reporting a reduction in post-partum liver TAG and blood FA and BHB concentrations in dairy cows that have undergone a controlled restriction before calving, despite an increase in precalving blood FA concentrations (Douglas *et al.* 2006; Loor *et al.* 2006; Janovick *et al.* 2011; Roche *et al.* 2005, 2015).

The apparent conflict between epidemiological or associative reports about the negative effect of precalving FA concentrations on reproduction outcomes and the positive effect of a managed reduction in DMI before calving on metabolic health

probably relates to the difference between correlation and causation. Where there is a positive correlation between pre- and post-calving blood FA concentrations, it is likely that whatever caused the precalving rise in blood FAs (i.e. the drop in DMI before calving) has also contributed to the rise in post-calving FAs and BHB in the blood (i.e. correlation). This hypothesis is supported by behavioural research undertaken by Huzzey *et al.* (2007) and Goldhawk *et al.* (2009), who reported that cows that got either metritis (Huzzey *et al.* 2007) or ketosis (Goldhawk *et al.* 2009) after calving had reduced time spent eating and DMI before calving. These observations indicate that the animals were 'unwell' before calving, but it was only with the stress of lactation that the diseases became clinical.

We contend that it is not the reduced DMI and elevated blood FAs before calving that cause post-calving disease and the negative effect on reproduction. It is more likely that there is a common cause for both the drop in precalving DMI (i.e. elevated precalving blood FAs) and the post-calving diseases. In fact, it is increasingly recognised that a controlled NEB in the weeks before calving (~90% metabolisable energy requirements) results in lower blood FAs and BHB and liver TAG after calving (Douglas *et al.* 2006; Loor *et al.* 2006; Janovick and Drackley 2010; Roche *et al.* 2015), increases the capacity of the liver for β-oxidation and gluconeogenesis while lowering ketogenesis (Loor *et al.* 2006) and improves the immune state (Lange *et al.* 2016). These features should, in fact, improve the metabolic state and increase the likelihood of a successful reproduction outcome in moderate to overconditioned cows (Thatcher *et al.* 2011). That said, the effect of a controlled NEB before calving on reproduction outcomes has not been evaluated. Further research is required to evaluate the role of precalving feeding level on the relationship among post-partum metabolic and immunological state and reproductive function.

Summary

The severity and duration of the post-calving NEB is important for reproduction, with higher blood FA and BHB and liver TAG concentrations all associated with a lower likelihood of pregnancy. Therefore, minimising the post-calving NEB to ensure blood FA and BHB concentrations are below 1.0 and 1.4 mM respectively (Raboisson *et al.* 2014) will likely increase reproductive success. Ensuring that cows are neither too thin nor too fat at calving is a very important component of managing BCS loss and blood FA and BHB concentrations. Furthermore, a controlled NEB before calving reduces the concentrations of FAs and BHB in the blood and TAG in the liver. These metabolic changes would be expected to increase the chance of a successful pregnancy, but further research is required to evaluate this management strategy at sufficient scale to measure reproduction outcomes.

Immune dysfunction and chronic inflammation

Broadly speaking, the immune system can be separated into two branches, the innate and adaptive responses, which, together, provide a coordinated response against infectious challenge. The innate system provides the fast first-line of defence using epithelial barriers, antimicrobial substances and various cell types, such as neutrophils, macrophages, dendritic cells and natural killer cells. The adaptive immune response differs from the innate immune response in that it takes time to develop (following antigen exposure), is highly specific and results in immunological memory. The adaptive immune system consists of antigen-presenting cells and antibody-producing B and T lymphocytes, which communicate with each other and cells of the innate system either through direct cell contact or via soluble factors (i.e. cytokines). Research over the past decade has revealed that the innate immune system is closely linked to the adaptive immune response, and that cells of the innate arm interact with and inform cells of the adaptive immune response (Mantovani *et al.* 2011).

Despite its necessity to life, the immune system is impaired during the transition period because of physical, hormonal and metabolic stresses associated with gestation, parturition and the onset of lactation (Kehrli *et al.* 1989; Cai *et al.* 1994; Mallard *et al.* 1997). Numerous studies have reported decreases in immune cell numbers in the blood (Park *et al.* 1992), reduced gene expression for immune components (Crookenden *et al.* 2016*a*; Madsen *et al.* 2002) and impaired chemotactic and phagocytic capabilities around parturition compared with mid- to late lactation (Shuster *et al.* 1996; Mehrzad *et al.* 2001; Heiser *et al.* 2015). For example, lymphocyte numbers decrease around parturition as a function of reduced proliferation (Kehrli *et al.* 1989).

The early lactation state of NEB is often characterised by impaired neutrophil function (Kehrli and Goff 1989; Kehrli *et al.* 1989; Burvenich *et al.* 2007; Crookenden *et al.* 2016*a*, 2016*b*), including trafficking, phagocytosis and killing capacity. In support of this, there is *in vitro* evidence indicating that a marked elevation in blood FA and BHB concentrations is a causative factor in immune suppression (Suriyasathaporn *et al.* 2000; Scalia *et al.* 2006). However, seminal research using mastectomised cows compared with intact cows indicated that the event of parturition and the associated metabolic and hormonal changes also contribute to the immune dysfunction (Kimura *et al.* 1999, 2002; Nonnecke *et al.* 2003), but that the lactation-induced NEB after calving sustains the suppression of immune cell function and the heightened inflammation response. Our own recent work has been aimed at identifying and understanding the underlying immune mechanisms (Heiser *et al.* 2015; Crookenden *et al.* 2016*a*, 2016*b*) and their interplay with nutrition and metabolism (Lange *et al.* 2016; Crookenden *et al.* 2017).

In addition to the immune system's defensive role, the transition period and, in particular, the early post-partum period are accompanied by an inflammatory state. This is, at least to some extent, a part of the natural physiological changes occurring in the periparturient period, but can also extend into a pathological condition (Bradford *et al.* 2015). The inflammatory process is characterised, in part, by excessive prostaglandin production; hence, one management strategy that has been hypothesised to reduce the chronic inflammation evident during the transition period and have potential benefits for reproductive function is the use of non-steroidal anti-inflammatory drugs (NSAIDs), with considerable interest in the role of salicylate (aspirin) in improving early lactation health, production and reproduction (Bradford *et al.* 2015).

Effects on reproductive function

Although there is limited information available in cattle, it is well established that immunity plays a direct and central role in successful reproduction (Walker *et al.* 2010; Hansen 2013; Fair 2015). Further, there is an indirect link between immunity and successful reproduction, because cows affected by mastitis and endometritis, a direct consequence of an under-performing immune system, are less likely to get pregnant (Sheldon *et al.* 2002; Hammon *et al.* 2006; Bromfield *et al.* 2015; Pinedo *et al.* 2016). Fair (2015) reported that preovulatory follicle differentiation and luteinisation are characterised by three phases of immune cell infiltration and that follicle development stage had a profound effect on the expression of genes in immune-related pathways in those tissues. Further, the immune system is integrally involved in follicle luteinisation and ovulation, sperm transport and maternal recognition of pregnancy (Fair 2015). Walker *et al.* (2010) reported that a key difference between identified fertile and subfertile strains of dairy cattle was in transcriptomic differences in immune tolerance genes in the endometrium during the pregnancy recognition phase. Therefore, a well-functioning immune system during the transition from pregnancy to lactation is essential to avoid the risk of infectious diseases, in the reinitiation of reproductive processes, during sperm transport and in preparing the uterus for pregnancy (Hansen 2013).

Improving post-partum immune function may involve nutritional strategies, such as managing NEB and blood FA and BHB concentrations post partum (see discussion above) and the use of micronutrients (vitamin and trace minerals) to promote antioxidant activity. Furthermore, strategic use of NSAIDs, immune enhancing agents and control of peripartum blood calcium concentrations (Sordillo and Raphael 2013; Bradford *et al.* 2015; Sordillo 2016) have all been investigated.

Use of micronutrients to enhance immune function

There is evidence that supplementation of the diet with antioxidants (e.g. vitamin E or α-tocopherol, selenium, vitamin A or β-carotene or retinol and vitamin C) is a practical way of enhancing immune cell function and decreasing inflammation (Sordillo *et al.* 2009). It was also recently reported that supplementation with rumen-protected omega-3 polyunsaturated fatty acids (PUFA) plus vitamin E from −21 through to +21 days relative to parturition resulted in lower post-partum blood concentrations of FA, BHB and bilirubin and greater α-tocopherol concentrations, indicating an overall improvement in liver function and attenuated inflammatory response post partum (Trevisi *et al.* 2011). Although ingested PUFA are substantially hydrogenated in the rumen, there is evidence that dietary supplementation with omega-3-rich oils during the peripartal period can enhance the concentrations of eicosapentaenoic acid and docosahexaenoic acid in liver phospholipids and adipose tissue TAG (Ballou *et al.* 2009), highlighting significant rumen escape of these FAs.

Use of NSAIDs

To improve the transition between pregnancy and lactation from an inflammatory state perspective, several studies have described the results of treating cows with different types of NSAIDs after calving; however, the results have been inconsistent. For example, Bertoni *et al.* (2004), in the first published study using an NSAID (acetylsalicylate) to dampen the peripartum inflammatory state, reported a positive effect of treatment during the first 5 days post partum on milk production and more favourable indices of inflammatory status (e.g. lower haptoglobin) during the first 4 weeks post partum. Farney *et al.* (2013) treated cows with acetylsalicylate just after calving for 1 week and, although milk production was not affected during the week of treatment, the NSAID-treated group produced more milk fat by Week 3 of lactation. From a reproduction perspective, Trevisi and Bertoni (2008) reported a significant increase in first service conception rates after salicylate treatment during the first 5 days after calving. However, treatment with salicylate before calving increased the severity of the inflammation, slowed post-partum recovery and decreased reproductive performance (Grossi *et al.* 2013). Inconsistent results have also been reported with other NSAIDs (Van Hecken *et al.* 2000; McDougall *et al.* 2009, 2016; Richards *et al.* 2009; Shwartz *et al.* 2009; Jeremejeva *et al.* 2012; Priest *et al.* 2013; Meier *et al.* 2014; Newby *et al.* 2017). There is evidence that under certain circumstances, but not others, treating early lactation cows with an NSAID may increase the chance of a successful pregnancy. Future research must focus on defining the exact criteria under which cows are likely to benefit from NSAID administration.

NSAIDs act to inhibit the biosynthesis of prostaglandins (Felson 2016) via inhibition of the two isoforms of prostaglandin endoperoxide H-synthase (PGHS; also known as cyclo-oxygenase (COX)-1 and -2). COX-1 is constitutively expressed in most mammalian cells (Smith *et al.* 1996), whereas COX-2 is usually induced by inflammatory stimuli (Williams *et al.* 1999; Smith *et al.* 2000). This mode of action makes NSAIDs a candidate management tool that could improve animal health through the transition period, including by improving uterine health. It is important to note that aspirin (salicylate) itself acts slightly differently from the other NSAIDs, because it binds irreversibly to the active site of the enzyme(s) and hence inactivates the enzyme(s). Other NSAIDs bind reversibly to the enzyme(s) to different extents. Moreover, aspirin can also have actions to suppress nuclear factor-kB activity, whereas the other NSAIDs act solely via inhibition of COX enzymes.

Use of immune-enhancing agents

Another potential strategy is the use of the commercially available immune stimulant, Imrestor (Elanco, Indianapolis, IN, USA). The active ingredient in Imrestor is the cytokine bovine granulocyte colony-stimulating factor (G-CSF), which regulates the growth and differentiation of neutrophil precursor cells within the bone marrow. As well as a peripartum immune restorative agent, the role of granulocyte–macrophage colony-stimulating factor (GM-CSF) has been extensively investigated by the Hansen laboratory in the US, establishing a role for GM-CSF in improving fertility *in vitro* and during embryo transfer (Loureiro *et al.* 2009; Block *et al.* 2011; Kannampuzha-Francis *et al.* 2015; Siqueira *et al.* 2017).

Recent studies have been conducted using a two-dose regime of 15 mg pegbovigrastim at ~7 days before the anticipated

calving date and then within 24 h of calving (Kimura *et al.* 2014; McDougall *et al.* 2017; Ruiz *et al.* 2017). Both McDougall *et al.* (2017) and Kimura *et al.* (2014) reported that the number of neutrophils increased markedly and remained elevated after calving in treated cows; the release of myeloperoxidase from the neutrophils was also greater, indicating both improved neutrophil number and function. In the study of Ruiz *et al.* (2017), cows treated with Imrestor had a 25% reduction in the incidence of clinical mastitis and untreated cows had 4.6% greater odds of having a retained placenta. Although the Imrestor-treated cows had 16.4% greater odds of being diagnosed with metritis within 21 days of calving and a 17.1% increase in the incidence of early metritis, there was a 5.8% greater chance of them being inseminated within 100 days after calving (Ruiz *et al.* 2017). These results indicate a direct effect of improving neutrophil number and function during the transition period on uterine health and possibly time to insemination. However, further research is required to determine the effects of Imrestor on uterine health and reproduction.

Summary

A functioning immune system as cows transition from pregnancy to a lactating state is a crucial component in preventing disease, initiating follicular development and in the actual fertilisation and maternal recognition stages of pregnancy. However, as part of the parturition event, the innate immune system becomes dysfunctional, with a reduction in the numbers and function of protective components, like neutrophils and lymphocytes, but exaggerated activity of inflammatory elements. Recent research efforts have targeted immune restorative products while dulling the post-partum inflammation with variable success. Further research is needed to understand the effects of enhanced immune function and reduced inflammation on reproduction outcomes.

Metabolic diseases associated with mineral deficiencies

Mineral deficiencies can be either primary or secondary and their effects on reproduction can be either direct or indirect. In general, primary mineral deficiencies result from a lack of a particular mineral in the diet. In comparison, secondary mineral deficiencies are the result of some other compound in the diet, often another mineral, which interferes with the absorption of the deficient element (e.g. sulfur, molybdenum, iron and calcium in the diet reduce the absorption of copper). Although a deficiency in many minerals can cause a reduction in reproductive function, the mineral element most notably 'deficient' during the transition period is calcium, with the metabolic disease resulting from its inadequacy termed hypocalcaemia or, more commonly, milk fever.

Hypocalcaemia can be the result of both a primary and secondary mineral deficiency, with low dietary magnesium and/or high dietary potassium reducing the absorption of calcium from the diet, a lack of dietary calcium after calving reducing the availability of calcium for absorption or a failure of the cow to adequately upregulate the eucalcaemic processes necessary to meet the calcium requirements of colostrogenesis; all will result in low blood calcium immediately before or after calving. Metabolic definitions of hypocalcaemia vary, but blood calcium concentrations <1.4 mM are generally regarded as clinically hypocalcaemic, whereas blood calcium <1.9–2.1 mM is defined as subclinically hypocalcaemic (Roche *et al.* 2013*a*).

Effects on reproductive function

Although there is only limited information of a direct effect of periparturient hypocalcaemia on reproductive function, indirect effects on smooth muscle function (Curtis *et al.* 1985; Martinez *et al.* 2014), dystocia and retained fetal membranes (Curtis *et al.* 1985), uterine involution (Heppelmann *et al.* 2015) and neutrophil function (Martinez *et al.* 2012, 2014) are well documented and would be expected to affect reproduction. Consistent with this effect, Caixeta *et al.* (2017) reported a delay in the resumption of ovarian activity after calving and a longer PPAI in hypocalcaemic cows, as well as a 50% reduction in pregnancy to first service (63% for eucalcaemic vs 31% for subclinical hypocalcaemic). Among cows at low risk of developing metritis, those with subclinical hypocalcaemia had a greater incidence of metritis (40.7%) than eucalcaemic cows (14.3%; Martinez *et al.* 2012). Therefore, we are confident that anything that increases the risk of hypocalcaemia is likely to reduce the likelihood of a successful pregnancy.

Although almost all cows undergo some degree of hypocalcaemia around parturition, there are effective management strategies to limit the severity of the drop in blood calcium and the duration of hypocalcaemic event.

Management of dietary magnesium

Magnesium intake is arguably the single greatest dietary factor determining the risk of milk fever (Lean *et al.* 2006; Roche and Berry 2006). Magnesium is essential for the efficient absorption and resorption of calcium; therefore, cows that have low blood magnesium around calving are more likely to get milk fever. In an analysis of 30 years of data from DairyNZ No. 2 Dairy, milk fever prevalence dropped from more than 10% to less than 5% following the introduction of precalving magnesium supplementation in the late 1970s–early 1980s (Roche and Berry 2006). In addition, Roche *et al.* (2002) reported that supplementation with magnesium sulfate and magnesium chloride was more effective than supplementation with magnesium oxide in maintaining periparturient eucalcaemia, despite a lack of effects of differences in dietary potassium and dietary cation–anion difference (DCAD). However, the use of these supplements is not practical on all farms.

Management of dietary calcium

Cows absorb only as much calcium as they require and it takes several days for a cow to alter the proportion of calcium she absorbs from her diet (Braithwaite 1974). When the cow calves and her requirements for calcium increase rapidly, she often cannot increase the proportion of dietary calcium absorbed sufficiently or quickly enough, and milk fever occurs.

Because of this relationship between dietary calcium and calcium absorption, traditional recommendations have been to feed a low-calcium diet before calving. In theory this will stimulate the cow to absorb a higher proportion of calcium from

her diet, such that when she calves and is fed a high-calcium diet she will absorb enough calcium to prevent milk fever. This strategy has been demonstrated to be effective on-farm (Wiggers *et al.* 1975). However, it is very difficult to reduce dietary calcium low enough during the dry period to sufficiently stimulate calcium absorption after calving to prevent milk fever. In comparison, Oetzel (1991) and Lean *et al.* (2006) noted that very low and very high levels of dietary calcium prepartum prevented milk fever, with the greatest risk of milk fever occurring between 0.5% and 2.0% DM calcium.

Compared with dry cows, the provision of supplementary calcium to colostrum cows, along with magnesium supplementation, will aid in the prevention of milk fever (Roche *et al.* 2002, 2003*a*).

Management of dietary potassium

Dietary potassium also contributes to the prevalence of milk fever, but the effect is not consistent across dairy systems. Research undertaken primarily in the US suggests that potassium is the primary nutritional factor contributing to milk fever through its effect on DCAD (Goff and Horst 1997*b*). Because of this, high-potassium forages should be minimised in the weeks before calving (NRC 2001). However, if this contraindication were appropriate for pasture-fed cows, 100% of cows would get milk fever due to the high potassium content of temperate pastures. In contrast, the incidence of milk fever in pasture-based herds is low (McDougall 2001), and New Zealand data indicated no difference in blood calcium around calving when cows were fed pastures varying from 3.3% to 4.2% DM potassium, the natural range evident in productive temperate pastures (Roche *et al.* 2002). This does not mean that potassium is unimportant. Potassium interferes with the absorption of magnesium in the rumen and, because magnesium is important for calcium absorption, thereby increases the risk of milk fever. However, potassium is secondary in importance to magnesium supplementation.

Management of dietary phosphorus

Feeds that are high in phosphorus increase the risk of milk fever by interfering with the renal activation of vitamin D (Kichura *et al.* 1982; Reinhardt *et al.* 1988). Therefore, feeds that are high in phosphorus (e.g. palm kernel extract, distillers grains) should be used with caution in the weeks before calving, particularly in herds prone to milk fever.

Dietary cation–anion difference

The DCAD is calculated from the amount of potassium, sodium, chlorine and sulfur in the diet, adjusting for their atomic mass and valence. The proportion of these minerals in the diet affects the acidity and alkalinity of the blood (blood pH; Stewart 1983). Blood pH affects calcium absorption from the intestine and bone calcium homeostasis (Roche *et al.* 2007*c*; van Mosel *et al.* 1993) through increased tissue sensitivity to parathyroid hormone and greater circulating concentrations of 1,25-$(OH)_2$-vitamin D (Goff *et al.* 2014). Blood pH drops significantly when DCAD is <0 mEq kg^{-1} DM and calcium absorption from the small intestine increases (Roche *et al.* 2003*b*, 2007*c*). Such a low DCAD is generally not achievable when high-potassium fresh forages are a significant part of the ration. However, lowering the DCAD through removal of potassium from the ration where practically possible facilitates the use of practical levels of anionic salts and will improve calcium absorption, thereby reducing the risk of milk fever in some circumstances.

Summary

Every cow undergoes a degree of hypocalcaemia at calving, but the severity and duration vary; it is these factors and not the presence of hypocalcaemia *per se* that appear to implicate hypocalcaemia in reproductive failure (Caixeta *et al.* 2017). There are numerous nutritional management strategies to maintain eucalcaemia during the transition from pregnancy to lactation, but management of dietary magnesium, calcium, potassium and phosphorus content during the weeks before calving can be particularly effective.

Exosomes as a potential future technology to enhance transition cow metabolic function

A new and exciting technology that could revolutionise both the identification of cows that have failed to transition well between the pregnant and lactating states or provide the vehicle for solution delivery lies in the understanding of exosomal signalling.

Exosomes are membrane-bound nanovesicles ranging from 30 to 120 nm in size. They are formed by inward budding of early endosomes to become multivesicular endosomes in the cytoplasm (Valadi *et al.* 2007). After fusion with the plasma membrane, they are released as exosomes into the extracellular space and/or into the circulation.

Exosomes can affect target cell function (e.g. membrane trafficking, cell proliferation and angiogenesis) following the uptake of their cargo (e.g. proteins, mRNA and microRNA (miRNA)), making them important intercellular messengers (Valadi *et al.* 2007). These characteristics of exosomes strengthen their potential utility in the treatment of diseases or subfertility, by using them for targeted delivery of drugs or silencing RNA (i.e. miRNA) to affected cells and organs (Sohel *et al.* 2013; Burns *et al.* 2016; Lee *et al.* 2016; Lopera-Vasquez *et al.* 2017; Shahabipour *et al.* 2017).

Due to the nature of their formation, exosomes provide important functional information on the propagating cell, including the oviduct (Lopera-Vasquez *et al.* 2016, 2017) and the uterus–conceptus complex (Burns *et al.* 2016). Recent studies of the roles of exosomes in cows have indicated that they have potential as indicators of fertility status (Mitchell *et al.* 2016) and disease state (Crookenden *et al.* 2016*b*). The mechanisms by which exosomes target specific cell types are still unclear, but further research will expose their prognostic and diagnostic potential.

In addition to a role as biomarkers, exosomes may be able to overcome some of the previously discussed insults caused by transition cow maladaptation. For example, exosome-derived miRNAs are differentially expressed in the follicular fluid of younger (<31 years) compared with older (>38 years) women, and are important regulators of gene expression during oocyte

maturation and both follicular and embryo development (Diez-Fraile *et al.* 2014; Lopera-Vásquez *et al.* 2016, 2017; Virant-Klun *et al.* 2016). da Silveira *et al.* (2014) have also reported the presence of exosomal miRNAs in equine follicular fluid that can regulate members of the transforming growth factor β/bone morphogenetic protein signalling pathway in granulosa cells. Exosomal signalling is also important during embryo implantation, for communication between the conceptus and the uterine wall, and exosomes have a role in immune regulation during early pregnancy, as demonstrated in ewes (Ruiz-González *et al.* 2015).

This research highlights exosomal formation, trafficking and actions in target cells as potential mechanisms for understanding and affecting reproduction in dairy cows. Therefore, exosomes may be used in future technologies to diagnose and/or improve disease status and fertility status in dairy cows. As mentioned earlier, exosomes have been exploited as a vehicle for drug delivery (Munagala *et al.* 2016), including anti-inflammatory agents (e.g. curcumin), to activated myeloid cells *in vivo* (Sun *et al.* 2010), as well as the delivery of chemotherapeutic agents to target and inhibit cancerous cell proliferation *in vivo* (Yao *et al.* 2013). Although studies of the utility of exosomes as carriers for therapeutic agents are in their infancy, the potential use of exosomes for targeted delivery of pharmaceutical and nutritional agents is a promising novel strategy to overcome subfertility resulting from transition period maladaptation.

Conclusions

Our knowledge of the biochemical and molecular processes associated with transitioning between pregnant and lactating states has increased markedly during the past 20 years (Roche *et al.* 2013*a*). Key problems associated with maladaptation during the transition can be grouped into diseases associated with energy metabolism, immune function and mineral deficiencies. Our knowledge of how transition period 'maladaptation' in all these areas negatively affects pre- and postovulatory reproductive processes has also increased, but there is further work to do to separate correlation from causation, thereby successfully targeting the root of the problem. There has already been a paradigm shift in how we approach precalving energy balance in optimally conditioned dairy cows to better manage post-calving NEB, metabolic disease and inflammatory state. Nevertheless, these approaches need to be tested at scale to determine whether they have a positive effect on reproductive variables. Further research into immune-enhancing compounds and NSAIDs will add greatly to the resolution of uterine remodelling and infectious diseases. The frontier lies in nanoparticle biomarkers and delivery mechanisms that will facilitate the targeting of specific tissues for support rather than a broad-spectrum approach to whole-animal medicine.

Conflicts of interest

The authors declare no conflicts of interest.

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