CSIRO PUBLISHING

Reproduction, Fertility and Development, 2013, **25**, 48–61
http://dx.doi.org/10.1071/RD12272

Associations between lipid metabolism and fertility in the dairy cow

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Abstract. Dairy cows mobilise body tissues to support milk production and, because glucose supplies are limited, lipids are used preferentially for energy production. Lipogenic activity is switched off and lipolytic mechanisms in adipose tissue increase through changes in the expression of several key enzymes. This results in a loss of body condition, together with high circulating concentrations of non-esterified fatty acids. Changes in the synthesis, secretion and signalling pathways of somatotrophic hormones (insulin, growth hormone, insulin-like growth factor 1) and adipokines (e.g. leptin) are central to the regulation of these processes. A high reliance on fatty acids as an energy source in the peripartum period causes oxidative damage to mitochondria in metabolically active tissues, including the liver and reproductive tract. The expression of genes involved in insulin resistance (*PDK4*, *AHSG*) is increased, together with expression of *TIEG1*, a transcription factor that can induce apoptosis via the mitochondrial pathway. Polymorphisms in *TFAM* and *UCP2*, two autosomal mitochondrial genes, have been associated with longevity in dairy cows. Polymorphisms in many other genes that affect lipid metabolism also show some associations with fertility traits. These include *DGAT1*, *SCD1*, *DECR1*, *CRH*, *CBFA2T1*, *GH*, *LEP* and *NPY*. Excess lipid accumulation in oocytes and the regenerating endometrium reduces fertility via reductions in embryo survival and increased inflammatory changes, respectively.

Additional keywords: antioxidants, energy balance, foam cells, mitochondria, reactive oxygen species.

Introduction

Lipid metabolism is an essential component of the homeostatic mechanisms activated when dairy cows start each lactation. The dietary energy intake at this stage is generally insufficient to meet the drain on the body's resources imposed by milk synthesis, so internal reserves are mobilised, leading to a loss of body condition. It may take a high-yielding animal many weeks to return to a positive energy balance (EB) status after calving (Wathes *et al.* 2007*a*; Grummer 2008). The EB is also compromised if dry matter intake is low due to disease or high environmental temperatures, both of which can impair appetite, or simply due to limited availability of adequate quality feed (Hayirli *et al.* 2002; Beever 2006). The general aim among most dairy farmers is to achieve a calving interval close to 365 days. This is particularly critical in systems based on a seasonal calving pattern and is still economically beneficial in all-year calving herds. In reality, however, typical calving intervals in many countries are over 400 days, and approximately 20% of cows are culled in each lactation, with failure to conceive the main reason (Brickell and Wathes 2011; Wu *et al.* 2012). There is a widespread acceptance that the fertility of modern cows is poor and needs to be improved in future by: (1) altering the genetic breeding goals to include non-production traits (Berry *et al.* 2003); and (2) improving management, in particular through better nutrition (Beever 2006). Both these goals can be assisted by an improved understanding of the mechanisms that link fertility to metabolism. This review focuses particularly on the internal control of lipid metabolism and the consequences of a high rate of lipolysis after calving. It will not consider the effects of feeding diets with differing lipid contents. This topic has been dealt with previously (Douglas *et al.* 2004; Grummer 2008).

Lipid metabolism

Lipids include cholesterol, phospholipids and triacylglycerols. These and their derivatives provide energy and are also essential components in a variety of endocrine and cell signalling pathways (Mattos *et al.* 2000; Wathes *et al.* 2007*b*). Adipose tissue is the main storage site, although lipids are also stored in other tissues, including muscle and liver, and are major components of all cell membranes. The amount of lipid present in the body at any one time is controlled by central and peripheral metabolic signals that regulate accumulation and mobilisation. The majority (>95%) of adipose tissue volume is composed of triglycerides stored within lipid droplets with a neutral lipid core and an outer phospholipid monolayer (Arner 2005). Adipose tissue secretes several adipokines, including leptin, resistin, tumour necrosis factor (TNF)-α and interleukin (IL)-6, which signal to the brain and peripheral tissues and contribute to the control of energy homeostasis (Vernon 2005).

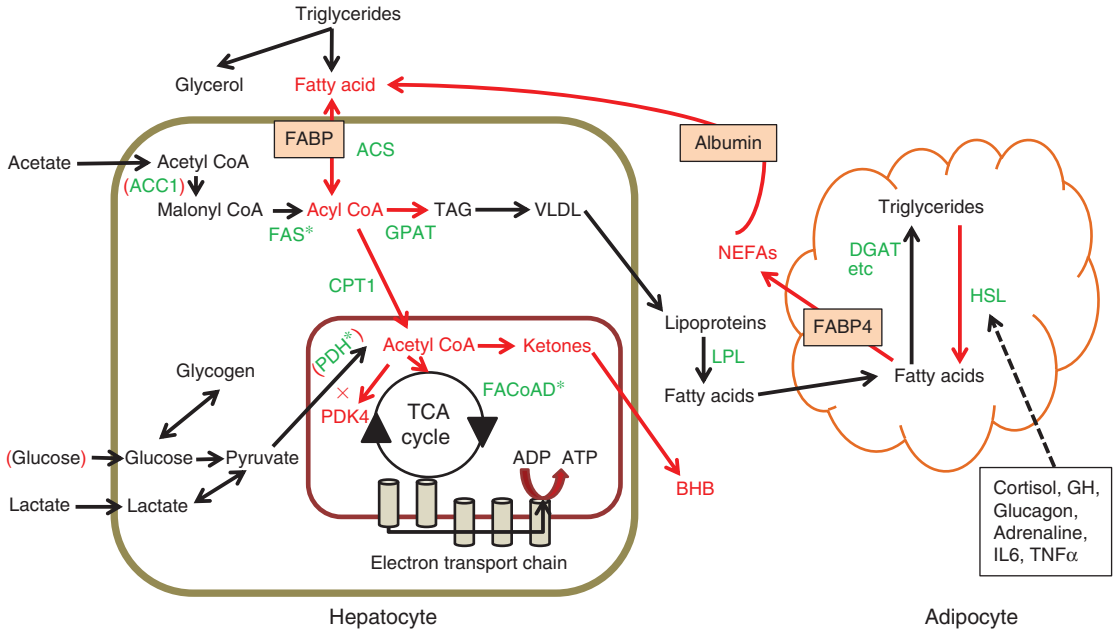

Fig. 1. Lipid metabolism in hepatocytes and adipocyte of lactating cows. Key enzymes involved are shown in green. The pathways upregulated during early lactation when cows are in negative energy balance are shown in red. Availability of circulating glucose and some enzymes is reduced (red brackets). The expression of those genes that are normally upregulated by insulin (indicated by asterisks) is decreased. Fatty acids taken up from the circulation by hepatocytes are used as an alternative energy substrate to glucose via β-oxidation in mitochondria. Excess acetyl CoA may be either partially oxidised to ketone bodies, such as β-hydroxybutyrate (BHB), re-esterified to triglycerides and stored, or exported as very low-density lipoprotein (VLDL). Because glucose is in limited supply, the use of pyruvate for energy metabolism is minimised by upregulation of pyruvate dehydrogenase kinase isozyme 4 (*PDK4*) and downregulation of pyruvate dehydrogenase (*PDH*). Within adipocytes, the uptake of fatty acids is reduced, lipogenic pathways are downregulated and lipolysis is increased by several endocrine factors that stimulate expression of hormone-sensitive lipase (HSL). This leads to an increase in the circulating concentrations of non-esterified fatty acids (NEFAs). ACC1, acetyl CoA carboxylase; ACS, acyl-CoA synthetase; CPT1, carnitine palmitoyl transferase 1; DGAT, diacylglycerolacyltransferase; FABP, fatty acid binding protein; FaCoAD, fatty acid CoA dehydrogenase; FAS, fatty acid synthase; GH, growth hormone; GPAT, glycerol-3-phosphate acyl transferase; IL-6, interleukin 6; LPL, lipoprotein lipase; TAG, triacylglycerol; TNF-α, tumour necrosis factor-α.

The biochemistry of lipid digestion has been reviewed in detail previously (Drackley *et al.* 2001; Vernon 2005). Lipogenesis occurs by two mechanisms. Circulating triglycerides in lipoproteins are hydrolysed by lipoprotein lipase, releasing fatty acids that are taken up by tissues. Once inside adipocytes, the fatty acids are converted to triacylgycerols by intermediary enzymes such as acyl CoA synthase, glycerol-3-phosphate acyl transferase (GPAT), phosphatidicacidphosphohydrolase and diacylglycerolacyltransferase (DGAT; Arner 2005; Vernon 2005). The second form of lipogenesis involves *de novo* synthesis of fatty acids. In ruminants this occurs following the uptake of acetate from the rumen under the control of acetyl CoA carboxylase (ACC1). The initial step in esterification before storage is then catalysed by GPAT (Vernon 2005). The mammary gland can also use β-hydroxybutyrate (BHB) for *de novo* fatty acid synthesis.

During lactation, lipogenic activity is reduced by decreasing both adipose tissue uptake and storage through reduced expression of ACC1 and lipoprotein lipase. At the same time, the release of fatty acids is stimulated by upregulation of lipase enzymes, including hormone-sensitive lipase (HSL). The final cleavage stage releases glycerol and non-esterified fatty acids (NEFAs). These are exported from the adipose tissue by fatty acid binding protein 4 (FABP4) and transported in the blood mainly bound to albumin (Martin *et al.* 2006; see Fig. 1). Circulating NEFA concentrations start to increase approximately 2 weeks before calving, peaking at 0–10 days post partum when EB reaches its nadir (Contreras and Sordillo 2011; Kawashima *et al.* 2012). These NEFAs consist mainly of saturated fatty acids, including palmitate (C16:0) and stearate (C18:0), and the monounsaturated fatty acid oleic acid (C18:1n9c; Contreras and Sordillo 2011).

Liver

The liver plays an essential role in lipid metabolism. The post partum rise in NEFAs provides an alternative energy substrate to glucose via β-oxidation. This, in turn, leads to a build up of acetyl CoA that may either be partially oxidised to ketone bodies, such as BHB, re-esterified to triglycerides and stored or exported as very low-density lipoproteins (Fig. 1). The NEFA concentration in blood reflects the extent of adipose tissue mobilisation, whereas circulating BHB provides an indication of fatty acid oxidation (Bauman and Currie 1980). When mitochondrial capacity is exceeded, oxidation is uncoupled

from ATP production and NEFAs can be oxidised in peroxisomes producing hydrogen peroxide and heat rather than ATP (Grummer 2008). Because the export capacity of the liver is limited, the build up of triglycerides in hepatocytes leads to the development of fatty liver (Vernon 2005). This happens to some extent in most dairy cows, peaking in the second week after calving (Drackley *et al.* 2001; Kruip *et al.* 2001; see Fig. 2*a*). During the peripartum period, concentrations of NEFA and BHB are correlated (Fig. 3); however, NEFA concentrations start to fall from approximately 1–2 weeks after calving while BHB concentrations continue to rise until about Week 6, as hepatic triacylglycerol is used for energy production (Wathes *et al.* 2007*c*). If the build up of triacylglycerol is excessive, this not only causes physical damage to the hepatocytes, but can also trigger apoptosis through endoplasmic reticulum stress and damage to mitochondrial membranes (Contreras and Sordillo 2011).

Milk production

During peak lactation, over 80% of the available glucose in the body is partitioned to the mammary gland for milk synthesis (Bell 1995; Hocquette and Bauchart 1999) and the majority of fats mobilised from adipose tissue contribute to milk fat synthesis (Grummer 1991). As genetic selection has increased milk volume, milk fat concentrations have remained at around 3.5%–4.0%, so the modern dairy cow must mobilise more tissue to meet this extra volume demand. The composition of milk fat is complex, containing over 500 different fatty acids, approximately 75% of which are saturated, 21% are monounsaturated and 4% are polyunsaturated (Mansbridge and Blake 1997). Most of the C4:0 to C14:0 are synthesised *de novo* from glycerol and free fatty acids whereas approximately 50% of C16:0 and the majority of longer-chain fatty acids are derived from the circulation from digestion or lipolysis from fat stores (Grummer 1991). The expression of enzymes involved in milk fat synthesis, such as stearoyl-CoA desaturase 1 (SCD1), can influence the amount of energy partitioned into milk and thus affect the overall EB status (Macciotta *et al.* 2008).

Endocrine regulation

Somatotrophic axis

The switch from lipogenesis to lipolysis is promoted by the changing endocrine environment during the peripartum period. Rapid tissue growth by the fetus in late gestation followed by lactogenesis drains the body of glucose (Bell 1995). This, in turn, lowers insulin secretion. High insulin concentrations promote anabolism, whereas low concentrations promote catabolism, thus influencing nutrient partitioning. Insulin signalling is mainly regulated downstream of the receptor, with high circulating lipid concentrations promoting peripheral insulin resistance. This is a mechanism to decrease glucose uptake into non-essential tissues during nutrient shortage (Drobny *et al.* 1984; White 2006).

Plasma growth hormone (GH) secretion peaks at calving or shortly thereafter, then gradually declines (Kawashima *et al.* 2007; Wathes *et al.* 2011). Although *GHR1A*, the liver-specific variant of the GH receptor (GHR), is downregulated during the periparturient period (Kobayashi *et al.* 1999; Fenwick *et al.* 2008*a*), GHRs continue to be expressed in adipose tissue. When blood concentrations of insulin are low, GH, together with catecholamines, promotes lipolysis (Vernon 2005). The timing of the switch to a catabolic state can be assessed by measuring circulating NEFA concentrations. In primiparous cows these generally start to rise some time before calving but, in older animals, the rise is coincident with the start of lactation (Wathes *et al.* 2007*c*; Fig. 3). The major downregulation of GHR1A is also responsible for the pronounced fall in circulating insulin-like growth factor (IGF) 1 concentrations, which begins before calving and typically reaches a nadir in the first week post partum (Taylor *et al.* 2004; Fenwick *et al.* 2008*a*).

Leptin

Leptin is a key hormone that contributes to the regulation of feed intake, energy partitioning and adipose tissue deposition during both short- and long-term changes in nutritional state (Ingvartsen and Boisclair 2001). Circulating leptin is derived mainly from white adipose tissue, although there may be local production in other tissues, such as muscle and placenta (Hoggard *et al.* 1997; Ramsay and Caperna 2009). Dairy cows have high leptin concentrations before calving that are proportional to their body condition score (BCS) and are also higher in heifers than cows. Concentrations fall at calving and then remain low even when the energy status has improved (Ingvartsen and Boisclair 2001; Wathes *et al.* 2007*c*; Fig. 3). It is thought that hypoleptinaemia may contribute to peripheral insulin resistance at this time (Ingvartsen and Boisclair 2001). There are also significant interactions between the intracellular actions of leptin and IGF, indicating cross-talk between their respective signalling pathways (Saxena *et al.* 2008).

Body condition score

Measurements of the BCS provide a useful proxy to assess lipid stores and mobilisation, although they focus on subcutaneous rather than deep tissue storage. Figure 3 illustrates the concomitant fall in BCS as milk yield increases and circulating NEFA concentrations rise. However, the fatty acid profile of NEFAs in the circulation more closely reflect the abdominal rather than subcutaneous fat stores, suggesting that this is more readily mobilised after calving (Hostens *et al.* 2012). Dry matter intake is the most important determinant of BCS, but the pre-calving diet and parity also influence the rate and extent of subsequent tissue mobilisation (Hayirli *et al.* 2002; Wathes *et al.* 2007*c*). Cows that are too fat (BCS 3.5 on a scale of 1–5) before calving suffer reduced appetite and rapid BCS loss after parturition. Conversely, cows that are too thin (BCS <2.5) may remain in poor condition subsequently, but without further BCS loss. Both scenarios are known to affect fertility. López-Gatius *et al.* (2003) combined results of 15 studies onto a scale of 1–5 and showed in a meta-analysis that loss of over 1 BCS unit after calving and a low BCS at first insemination both led to a significant increase in days open. Others have shown that oestrous cycles are unlikely to resume until after the nadir in bodyweight is reached (e.g. Butler 2001; Westwood *et al.* 2002; Wathes *et al.* 2007*d*). The BCS is often used as an assessment of energy status

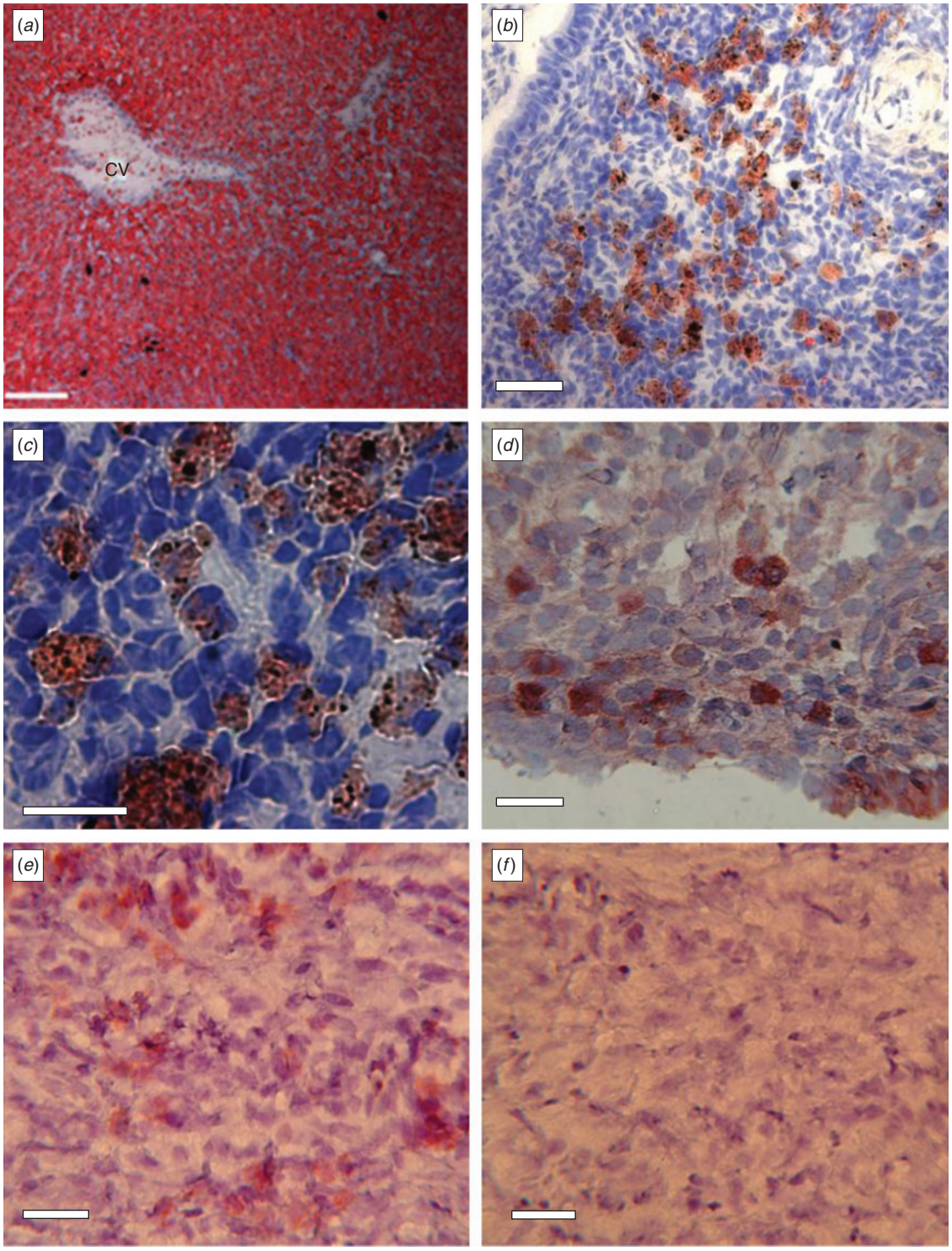

Fig. 2. All sections are taken from dairy cows in severe negative energy balance at 14 days post partum. (*a–c*) Oil Red O- and haematoxylin-stained sections. (*a*) Liver sample showing extensive accumulation of lipid around the central vein (CV). (*b*) Endometrial tissue showing lipid accumulation in the subepithelial stroma. (*c*) Higher magnification of (*b*) showing individual cells full of lipid. These have the appearance of foam cells. (*d*) Immunohistochemistry of the endometrium stained with an antibody against CD172a, a marker for granulocytes and macrophages. (*e*) Immunohistochemistry of the endometrium stained with an antibody against CD14, a macrophage marker. (*f*) Control section for (*e*) with the primary antibody omitted. Scale bars = 100 μm (*a*), 50 μm (*b*) and 25 μm (*c–f*).

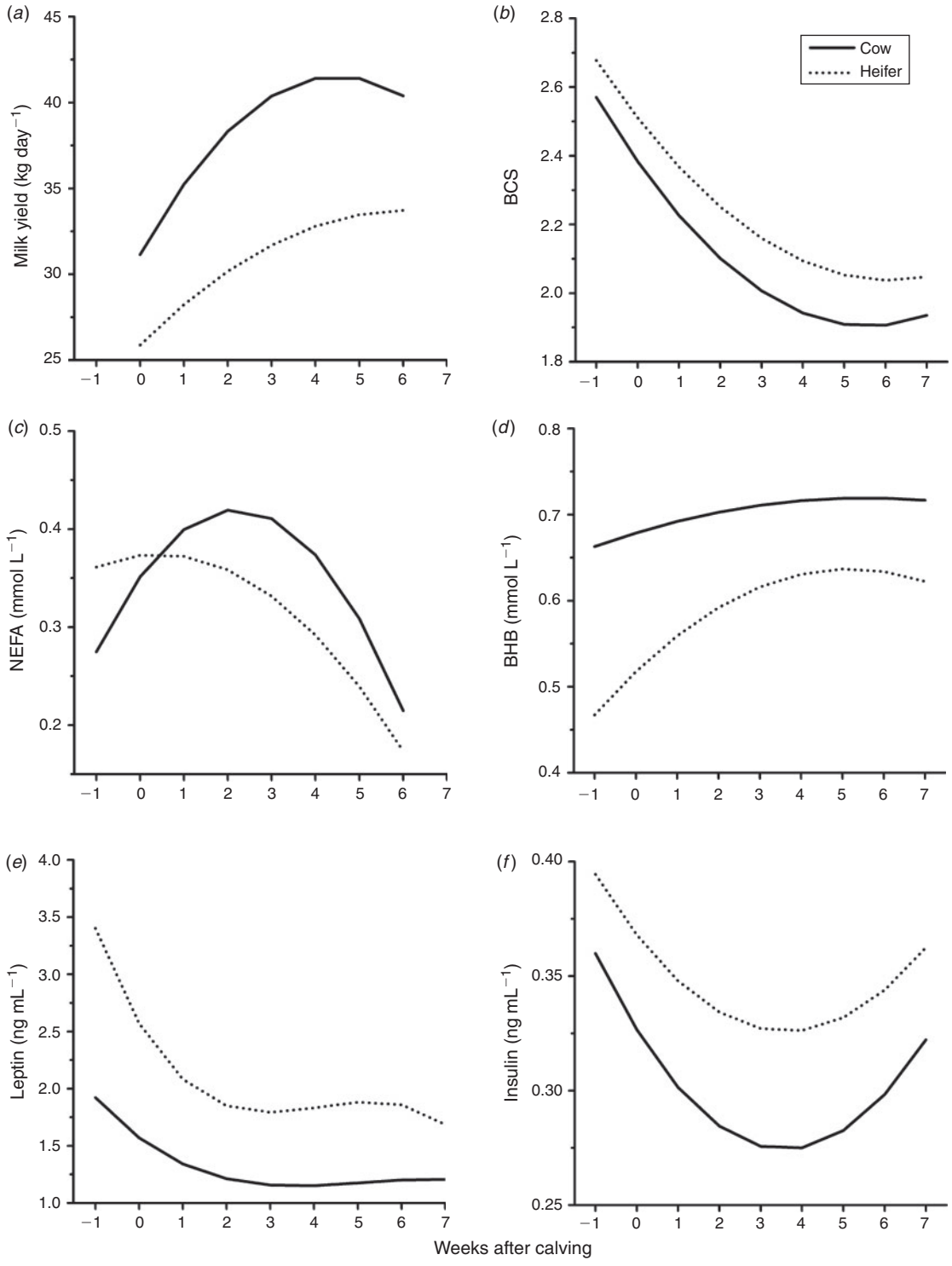

Fig. 3. Changes in milk yield, body condition score (BCS), metabolites (non-esterified fatty acids (NEFAs) and β-hydroxybutyrate (BHB)) and metabolic hormones (leptin and insulin) during the peripartum period in dairy cows. Graphs show the predicted least mean square concentrations for first calving animals (heifers; $n = 188$) and multiparous cows ($n = 312$) that were each sampled at −1, +1, +4 and +7 weeks with respect to calving. Data are from Wathes *et al.* (2007*c*).

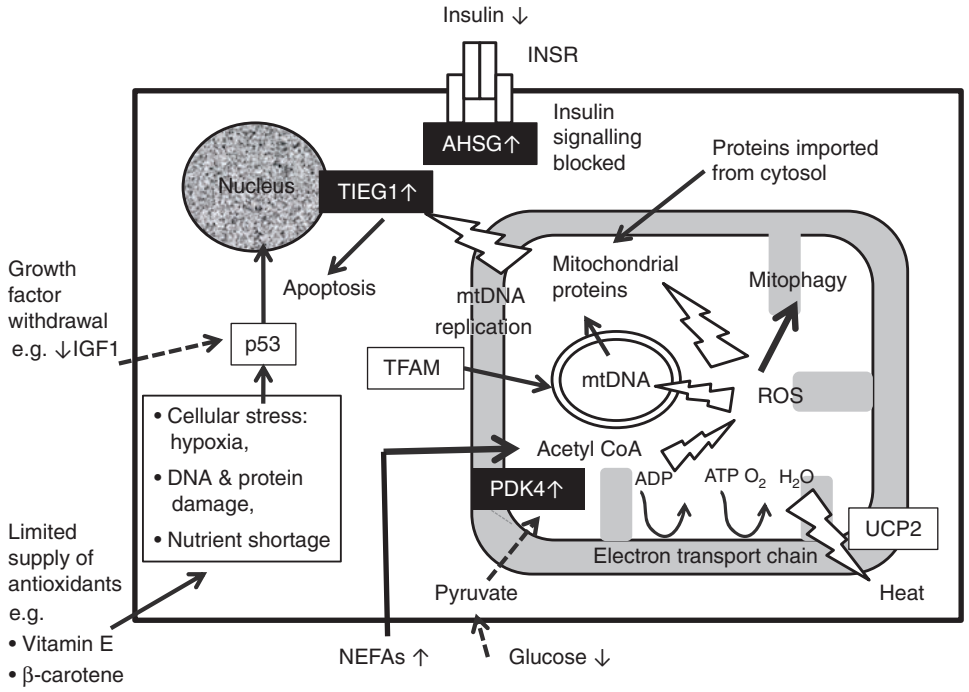

Fig. 4. Summary diagram showing potential effects of negative energy balance on mitochondria. The supply of glucose is limited, so increased expression of pyruvate dehydrogenase kinase isozyme 4 (*PDK4*) promotes use of non-esterified fatty acids (NEFAs) for energy production via the electron transport chain. This results in increased production of reactive oxygen species (ROS). These, in turn, damage both mitochondrial proteins and DNA. Key antioxidants, such as vitamin E and β-carotene, are also in limited supply during early lactation. Damaged mitochondria are removed by mitophagy or apoptosis. The balance towards apoptosis is promoted by reducing insulin-like growth factor (IGF) 1 concentrations and downregulation of insulin signalling pathways, in part via increased expression of α2-HS-glycoprotein (*AHSG*). This leads to growth factor withdrawal. Increasing cellular stress increases apoptosis through the p53 pathway and by upregulation of the transcription factor transforming growth factor-beta-inducible early growth response protein 1 (*TIEG1*). Transcription factor A, mitochondrial (*TFAM*) influences mitochondrial replication, whereas uncoupling protein 2 (*UCP2*) may be important for the attenuation of ROS production by transporting protons across the inner mitochondrial membrane, leading to extra heat production. Polymorphisms in both *TFAM3* and *UCP2* have been associated with reduced longevity in dairy cows. INSR, insulin receptor.

in genetic studies because it is a heritable trait that is negatively correlated with fertility traits such as pregnancy rate and calving interval (Pryce *et al.* 2002; Berry *et al.* 2003).

Mitochondria and oxidative stress

Mitochondria are critical centres of energy conversion and metabolism that dictate the energetic balance of the cell and are the main site of intracellular oxygen consumption. A mechanism to maintain an adequate population of healthy mitochondria is essential to cell survival. Autophagy is a housekeeping process by which cells remove damaged organelles, intracellular pathogens and misfolded or aggregated proteins through lysosomal-dependent machinery (Glick *et al.* 2010). This is a normal part of homeostasis that can be triggered by a variety of factors, including nutrient shortage (Dolganiuc *et al.* 2012). Mitophagy is a specific form of autophagy that involves breakdown of mitochondria, promoting maintenance of a functional mitochondrial population and providing additional nutrients to the cell in times of shortage (Mammucari and Rizzuto 2010). Mitochondria are particularly vulnerable to damage because they produce large amounts of reactive oxygen species (ROS; hydrogen peroxide (H_2O_2) and superoxides) as a by-product of the electron transport chain. If ROS concentrations become excessive, both proteins and DNA are damaged to such an extent that changes within the mitochondria promote apoptosis of the whole cell. One trigger for this is increasing activity of p53, which acts as a sensor of intracellular stress signals, including DNA damage, hypoxia and nutrient shortage. At the same time, growth factor withdrawal may remove protective anti-apoptotic effects (Mammucari and Rizzuto 2010; see Fig. 4). Oxidative stress also damages cell membranes by causing lipid peroxidation (Malin *et al.* 1999).

In the dairy cow, a variety of factors are likely to promote mitochondrial damage in the early post partum period. Lactogenesis requires a significant increase in metabolism, and fatty acids become a major source of energy at this time because the glucose supply is limited (Bauman and Currie 1980). At the same time a variety of antioxidant defence mechanisms against

ROS may become compromised. Antioxidant enzymes include superoxide dismutase (SOD); this converts the superoxide anion to H_2O_2, after which catalase and glutathione peroxidase (GPx) further degrade the H_2O_2 to water. Bernabucci *et al.* (2005) showed that plasma SOD concentrations fell after calving, whereas those of GPx increased. The extent of the changes was related to the circulating NEFA and BHB concentrations and cows with higher BCS or greater BCS loss were more sensitive to oxidative stress. Non-enzymatic antioxidants include vitamin C (ascorbic acid), vitamin E (α-tocopherol), glutathione and β-carotene (Agarwal *et al.* 2012). β-Carotene acts both as a precursor of vitamin A and independently to enhance host immunological defence mechanisms, but circulating concentrations fall around calving to a minimum at 1–2 weeks post partum (Kawashima *et al.* 2012). Some, but not all, studies have reported that supplementing the diet with β-carotene around this time improves fertility and reduces markers of uterine inflammation (Kaewlamun *et al.* 2011; Kawashima *et al.* 2012). Concentrations of vitamin E, another fat-soluble vitamin with antioxidant properties, may also be inadequate around calving, with a meta-analysis showing that vitamin E supplementation could reduce the risk of retained fetal membranes (Bourne *et al.* 2007).

We have previously reported an experiment in which gene expression arrays were used to assess the effects of severe negative energy balance (NEB) on the oviduct, uterus and liver of the post partum cow (Fenwick *et al.* 2008*b*; Wathes *et al.* 2009; McCarthy *et al.* 2010). Although there were many tissue-specific effects, some genes showed a large increase in expression across all tissues, including pyruvate dehydrogenase kinase isozyme 4 (*PDK4*), which encodes an enzyme located in the matrix of the mitochondria that is upregulated in response to an increased lipid supply, inactivating the pyruvate dehydrogenase complex and helping to conserve glucose by limiting the conversion of pyruvate to acetyl-CoA (Holness and Sugden 2003). Another gene with higher expression during NEB was transcription factor transforming growth factor-beta-inducible early growth response protein 1 (*TIEG1*). This is a member of the three zinc finger family of Krüppel-like transcription factors that induces apoptosis via the mitochondrial pathway and inhibits cell proliferation (Jin *et al.* 2007). The simultaneous upregulation of these two genes in both the liver and reproductive tract support the concept that the dependence of the post partum dairy cow on NEFAs as a major energy source may predispose towards mitochondrial damage in a variety of tissue types (Fig. 4).

Genetic control of lipid metabolism and its association with fertility

The control of lipid metabolism is vital in both the dairy and beef cattle industries, so many studies have investigated associations between polymorphisms in genes associated with lipid metabolism and commercially important traits to use this information in breeding programmes. In dairy cows, the traits of interest have focused on milk production and quality (e.g. fat and protein yield), whereas in beef cattle meat yield and quality traits such as subcutaneous fat depth and marbling have been studied (see Table 1 for references). In both dairy and beef breeds, genes associated with the endocrine control of growth, body condition and energy metabolism (e.g. IGF1, GH, leptin and their receptors) have also been investigated. Due to the established links between metabolism and fertility, all these genes may reasonably be expected to influence fertility traits too. In the main, however, the associations with fertility have been less significant than those with milk and meat production. Results from several such studies are summarised in Table 1.

Mitochondrial genes

In our own work we have investigated associations of single nucleotide polymorphisms (SNPs) in selected genes with milk production, fertility and survival traits in a starting population of 509 Holstein-Friesian heifers from 19 UK dairy farms (Brickell *et al.* 2009*a*, 2009*b*) Fertility was assessed in both nulliparous (i.e. non-lactating) heifers and in the same animals in their first and second lactations. The SNP having the strongest association with fertility traits was in the gene transcription factor A, mitochondrial (*TFAM*). *TFAM* is an autosomal mitochondrial gene that encodes a histone-like protein essential for the transcription and replication of mitochondrial DNA (Jiang *et al.* 2005). Cows that were GG homozygotes for the SNP *TFAM3* not only produced less milk than the AG heterozygotes, but they also had worse fertility, being less likely to conceive and having a 24 day longer calving-to-calving interval (Clempson *et al.* 2011*a*). The GG homozygotes were also more likely to be culled or die prematurely, with fewer surviving into a third lactation. The AA homozygotes for *TFAM3* had lower milk production and slightly worse fertility than the heterozygotes.

An SNP in another autosomal mitochondrial gene, namely uncoupling protein 2 (*UCP2*), was also associated with survival, with more GG than CG cows being culled before third calving (64% vs 37%, respectively; $P < 0.05$; Clempson *et al.* 2011*a*). The UCPs contribute to the regulation of energy metabolism and the attenuation of ROS production by transporting protons across the inner mitochondrial membrane, leading to the production of heat (Echtay 2007). It is possible that the SNPs studied reflect modifications to the TFAM and UCP2 proteins that affect the ability of the mitochondria to adapt to the increased input of NEFAs as the main energy supply at the start of lactation. This could potentially increase ROS production, resulting in cell damage a variety of tissues, thus decreasing both fertility and longevity (Fig. 4).

Leptin

As outlined above, leptin is a key signalling molecule derived mainly from adipocytes that is known to influence both metabolism and fertility. In our study leptin SNPs were more strongly associated with fertility than with milk production (Clempson *et al.* 2011*b*). Of five different leptin SNPs investigated, all were significantly associated with some aspect of fertility, including age at first service and services per conception in heifers, age at first calving, days to conception, proportion in-calf at 100 days post partum and calving interval. In contrast, only one SNP in our study (*A59V*) was associated with milk yield, although other studies have found some associations

Table 1. Summary of genes involved in lipid metabolism and their single nucleotide polymorphism associations with traits for milk production, body fat composition and fertility

DAG, diacylglycerol; TAG, triacylglycerol; mtDNA, mitochondrial DNA; ROS, reactive oxygen species; HPA, hypothalamic–pituitary–adrenal; Y, yes; N, no; SNP, single nucleotide polymorphism

Gene	Full name	Primary function(s)	SNP associations found[A]			References
			Milk	Body fat	Fertility	
DGAT1	Acyl-CoA diacylglycerolacytransferase	Converts DAG to TAG Major regulator of milk fat %	Y	Y	Y/N	Grisart *et al.* (2002); Ashwell *et al.* (2004); Kaupe *et al.* (2007); Schennink *et al.* (2007); Banos *et al.* (2008); Demeter *et al.* (2009); Gill *et al.* (2009); Oikonomou *et al.* (2009)
SCD1	Stearoyl-CoA desaturase 1	Rate-limiting enzyme in conversion of saturated to monounsaturated fatty acids These are substrates for triglycerides, esters, membrane phospholipids and milk fat	Y	Y	N	Taniguchi *et al.* (2004); Moioli *et al.* (2007); Ohsaki *et al.* (2007); Jiang *et al.* (2008); Macciotta *et al.* (2008); Schennik *et al.* (2008); Cecchinato *et al.* (2012)
DECR1	2,4-Dienoyl CoA reductase 1	Nuclear-encoded mitochondrial enzyme involved in β-oxidation Controls rate of fatty acid metabolism for energy production	Y	Y	Y	Marques *et al.* (2009); Clempson (2010)
FABP4	Fatty acid binding protein 4	Lipid binding in adipocytes Influences intracellular lipid trafficking	Y	Y	N	Michal *et al.* (2006); Cho *et al.* (2008); Barendse *et al.* (2009); Lee *et al.* (2010); Clempson (2010)
TFAM	Transcription factor A, mitochondrial	Histone-like protein essential for transcription and replication of mtDNA	Y	Y	Y	Jiang *et al.* (2005); Clempson *et al.* (2011*a*)
UCP2	Uncoupling protein 2	Transports protons across the inner mitochondrial membrane, contributing to the regulation of energy metabolism and the attenuation of ROS production	N	Y	Y	Sherman *et al.* (2008); Clempson *et al.* (2011*a*)
CRH	Corticotropin-releasing hormone	Released from appetite control centre in the brain Regulates HPA axis to regulate energy mobilisation in response to stress	Y	Y	Y	Wibowo *et al.* (2007); Clempson (2010)
FGF8	Fibroblast growth factor 8	Regulator of other genes influencing adiposity	N	Y	N	Marques *et al.* (2009); A. M. Clempson, unpublished observations
CBFA2T1	Core binding factor, runt domain, α-subunit 2, translocated to 1	Transcription factor highly expressed in adipose tissue, thought to regulate lipid metabolism and obesity	Y	Y	Y	Marques *et al.* (2009); A. M. Clempson, unpublished observations
GH	Growth hormone	Regulator of growth and body composition	Y/N	Y	N	Balogh *et al.* (2009); Mullen *et al.* (2011)
GHR	Growth hormone receptor		Y	N	Y/N	Viitala *et al.* (2006); Gill *et al.* (2009); Oikonomou *et al.* (2009); Clempson (2010)
LEP	Leptin	Adipokine that regulates lipid metabolism and fertility	Y	Y/N	Y	van der Lende *et al.* (2005); Banos *et al.* (2008); Gill *et al.* (2009); Clempson *et al.* (2011*b*); Woronuk *et al.* (2012)
LEPR	Leptin receptor		Y	?	N	Banos *et al.* (2008); Clempson *et al.* (2011*b*)
NPY	Neuropeptide Y	Neurotransmitter that controls appetite and energy homeostasis	Y	Y	Y	Sherman *et al.* (2008); Clempson *et al.* (2011*b*)

[A]Milk traits investigated in different studies include aspects of yield and composition in dairy cows; body fat traits include relevant aspects of fatness and carcass composition in beef cattle; fertility traits are from those studied in lactating dairy cows.

of leptin SNPs with milk yield and composition (van der Lende *et al.* 2005; Banos *et al.* 2008). An SNP in neuropeptide (NPY) was also significantly associated with the in-calf rate at 100 days (Clempson *et al.* 2011*b*). NPY is a neurotransmitter that controls appetite and energy homeostasis and is a key regulator of leptin activity in the hypothalamus (Bahar and Sweeney 2008). However, the leptin receptor SNP *T945M* was not associated with either milk production or fertility traits, in agreement with a previous study (Banos *et al.* 2008).

Collectively, these results suggest that leptin polymorphisms present in dairy cow populations can influence both milk production and fertility. The question arises as to whether the effect of leptin on fertility follows on indirectly from an influence on lipid metabolism and body condition. Although this is indeed possible, our data showed more effects on fertility than on milk, and this included significant relationships between polymorphisms and fertility in non-lactating heifers. Furthermore, milk production was accounted for in the model used to test the associations with fertility. This supports the importance of a direct role of leptin on reproduction. In particular, leptin treatments *in vitro* can contribute to the regulation of ovarian steroidogenesis, showing synergistic actions with IGF1 (Spicer 2001; Nicklin *et al.* 2007). Leptin can also influence oocyte maturation and fertilisation rate (Boelhauve *et al.* 2005) and gonadotrophin secretion, although in ruminants this latter action only seems to be important during very severe undernutrition (Zieba *et al.* 2005).

Lipid metabolism

Other SNP we investigated in genes with known effects on lipid metabolism all, with the exception of fibroblast growth factor 8 (*FGF8*), showed some significant associations with milk production (2,4 dienoyl CoA reductase 1 (*DECR1*), core binding factor, runt domain, α subunit 2, translocated to 1 (*CBFA2T1*), corticotrophin releasing hormone (*CRH*), acyl-CoA diacylglycerolacytransferase (*DGAT1*), fatty acid binding protein 4 (*FABP4*), stearoyl-CoA desaturase 1 (*SCD1*) and growth hormone receptor (*GHR*)) (Clempson 2010; see Table 1). There were also some small associations ($P < 0.05$) with various fertility traits: SNPs in *DECR1*, *CBFA2T1* and *CRH* were all associated with days to first service and *DECR1* was also associated with calving interval. For example, animals in their second lactation with the CC genotype for *DEC7* produced 1059 ± 432 kg more milk than the heterozygotes, but also had a 20 ± 7 day longer calving interval. The gene for *DECR1* encodes the mitochondrial enzyme 2,4dienoyl CoA reductase 1, which is involved in β-oxidation and controls the rate of fatty acid metabolism for energy production. For these genes, it is more likely that effects on fertility were secondary to changes in lipid metabolism that influenced the amount of milk production in early lactation, thus altering the extent of lipid mobilisation and energy balance deficit.

The effects of *DGAT* polymorphisms have been well established with the *F279Y* SNP likely to be the causative mutation, because this was shown to affect triglyceride synthesis *in vitro* (Grisart *et al.* 2002). Although we did not find differences in fertility associated with this SNP, others have reported changes in pregnancy rate (Ashwell *et al.* 2004; Kaupe *et al.* 2007) and non-return rate (Demeter *et al.* 2009). Because changes in pregnancy rates were also associated with alterations in BCS, peripheral glucose and NEFA concentrations, an indirect effect of DGAT on fertility via alterations in EB seems most likely (Banos *et al.* 2008; Oikonomou *et al.* 2009). Unlike us, Oikonomou *et al.* (2009) also found associations between the GHR SNP *F279Y* and some fertility traits. However, Balogh *et al.* (2009) failed to find any associations between a polymorphism in the GH gene and time of first post partum ovulation, BCS loss or milk yield in the first month after calving. Therefore, although GH clearly has a major role in lipid mobilisation, differences in fertility between animals do not seem to be directly attributable to differences in GH or GHR genotype within existing dairy cow populations.

Effects of lipid metabolism on the reproductive tract

Ovary

The oocytes that are ovulated 2–4 months after calving, at a time when breeding is desired, have undergone their earlier stages of maturation during the nadir of NEB. Several lines of evidence suggest that this may compromise oocyte quality. Snijders *et al.* (2000) found that oocytes derived from high-yielding cows formed fewer blastocysts than those from medium genetic merit animals. This was related, in part, to BCS, with a lower success rate when the BCS of the donor was <2.5. Leroy *et al.* (2008) reviewed some of the mechanisms by which high-fat diets may compromise early embryo development. *In vitro* experiments have shown that both oocytes and embryos accumulate fatty acids from their environment (Kim *et al.* 2001; Fair 2003). Although the stored triglycerides provide an important energy source, excessive accumulation impairs mitochondrial function and makes them more vulnerable to oxidative stress, as discussed above (Rizos *et al.* 2003). Wrenzycki *et al.* (2000) showed that the nutritional regimens to which donor heifers were exposed altered the abundance of SOD transcripts in embryos recovered after superovulation. Using an *in vitro* approach, Marei *et al.* (2012) found that bovine oocyte maturation in the presence of added linoleic acid influenced the distribution of mitochondria in the cytoplasm, decreased the mitochondrial inner membrane potential and increased levels of ROS. Because elevated NEFA concentrations can be cytotoxic, Van Hoeck *et al.* (2011) went on to demonstrate that exposure to high concentrations of NEFAs, in particular oleic acid, palmitic acid and stearic acid, during oocyte maturation reduced subsequent embryo quality, measured in terms of cell number, gene expression and apoptotic cell ratio. Similar adverse effects were produced by culturing bovine zygotes with serum derived from heifers fed a high-fat diet supplemented with palm oil (Leroy *et al.* 2010). Together, these experiments suggest that a period of extreme NEB experienced after calving, which will inevitably be accompanied by elevated circulating NEFA concentrations, is likely to impair oocyte quality. This, in turn, will reduce conception rates through excessive lipid build up, ROS production and mitochondrial damage.

Uterus

A cow cannot conceive again after calving until her reproductive tract has recovered sufficiently to support another pregnancy.

An impaired uterine environment is likely to be a major contributor to the higher incidence of early embryonic death found in repeat-breeder cows (Hill and Gilbert 2008). Uterine involution requires a considerable amount of tissue remodelling as the size reduces back towards a non-pregnant level and the extensive damage at the surface of the caruncles caused by placental separation needs to be repaired (Gier and Marion 1968; Llewellyn *et al.* 2008). At the same time, the majority of uteri become infected with pathogenic organisms (Sheldon *et al.* 2006) while the immune status is impaired during the peripartum period (Cai *et al.* 1994; Mallard *et al.* 1998). This makes cows less able to withstand microbial infections and approximately 15% of all dairy cows go on to develop endometritis (Sheldon *et al.* 2006).

The reduction in the ability to mount an effective immune response is influenced by the extent of NEB around calving (Pyörälä 2008; Wathes *et al.* 2009) because it is an energetically demanding process (Fox *et al.* 2005). Acute infections cause local insulin resistance (Drobny *et al.* 1984) and we have shown previously that α-2-HS-glycoprotein (*AHSG*) and pyruvate dehydrogenase kinase, isozyme 4 (*PDK4*), two genes implicated in insulin resistance, are upregulated in the endometrium when cows are in severe NEB, with the expression of both genes showing a significant positive correlation with circulating NEFA concentrations (Wathes *et al.* 2011). The main site of AHSG synthesis is the liver, with protein production increasing in response to fat accumulation, which, in turn, inhibits insulin receptor signalling (Stefan *et al.* 2006). The enzyme PDK4 contributes to the regulation of glucose metabolism, as discussed above. Circulating IGF1 concentrations are very low at this time (Taylor *et al.* 2004) and the local IGF system in the uterus is also altered (Llewellyn *et al.* 2008; Wathes *et al.* 2011). These changes may also contribute to delayed recovery of the endometrium because IGF1 has a positive effect on tissue repair mechanisms (Mourkioti and Rosenthal 2005).

As part of the mechanism to withstand infection, circulating monocytes are attracted into damaged tissues by chemotactic signals and then differentiate into macrophages or dendritic cells. Macrophages preferentially take up oxidised low-density lipoprotein (ox-LDL) via scavenger receptors, resulting in their transformation into foam cells. In humans this process is particularly associated with the development of atherosclerosis following endothelial damage; this initiates adhesion of monocytes to the endothelium before migration into the arterial wall (Bobryshev 2006). Foam cell formation in humans is also stimulated by the presence of the pathogen *Chlamydia pneumonia* (Kalayoglu and Byrne 1998). The development of foam cells initially functions as a protective mechanism by removing cytotoxic and inflammatory ox-LDL via degradation in lysosomes, but excessive accumulation promotes the development of atherosclerotic plaques. The build up of lipoprotein also deranges normal macrophage function (Tabas *et al.* 2007).

Foam cells have been identified previously in cervical smears and endometrial biopsies of women with uterine pathology (Silver and Sherman 1998). Therefore, we investigated the presence and localisation of lipids in the bovine endometrium during the early post partum period. At around 14 days post partum, some cows had very high levels of lipid accumulation, mainly in the subepithelial stroma (Fig. 2*b*). Close examination revealed the presence of cells within this layer with the appearance of foam cells (Fig. 2*c*). This was supported by staining with CD172a and CD14, which label monocytes/granulocytes and macrophages, respectively (Fig. 2*d*, *e*). The high circulating NEFA concentrations at this time, together with poor oxidative status, are likely to cause peroxidative damage to lipids. These results suggest that the tissue damage after calving, bacterial infection and an influx of monocytes contribute to a build up of lipid and formation of foam cells in the endometrium in the post partum period. This is supported by studies that have found increased expression of genes associated with Nrf-2 mediated oxidative stress in the endometrium of post partum cows in severe NEB (e.g. *SOD2*, 2.15-fold increase; $P < 0.0001$), whereas *GSTA1*, which contains antioxidant response elements, was downregulated (0.5-fold decrease; $P < 0.0001$; Wathes *et al.* 2009).

Conclusions

It is clearly established that as cows enter NEB after calving there is a switch towards the increasing use of fatty acids as an energy source to conserve limited supplies of glucose. This is promoted by several endocrine signalling pathways: IGF1 and leptin concentrations fall, insulin signalling is blocked and GH and catecholamine secretion promote lipolysis. The rate of body tissue mobilisation is influenced by energy input (dry matter intake), energy stores (BCS) and energy output (milk production) in the critical period around calving. Genetic selection for hormones and enzymes that promote tissue mobilisation and milk synthesis during this period can impact on fertility indirectly as the energy deficit post partum is worsened. Several metabolic signalling pathways have evolved in mammals to relay this information to the reproductive system to block reproductive processes when energy is in short supply. At the same time, the high circulating concentrations of NEFAs, the increasing utilisation of fatty acids by mitochondria and a possible insufficiency of antioxidants can have additional adverse consequences throughout the body. In particular, this situation will increase the production of ROS, causing mitochondrial damage and perhaps triggering apoptotic mechanisms. Although these changes are likely to impact on most metabolically active cells, we have presented evidence for damage to oocytes and the endometrium that contributes to the reduction in fertility in dairy cows in which energy supplies are compromised by either high milk yields or peripartum disease.

Acknowledgements

The authors are very grateful to Defra, DairyCo, the Wellcome Trust, Merial Animal Health and Biotechnology and Biological Sciences Research Council (BBSRC), who have funded various aspects of our own work included here. The work on NEB in tissue samples from post partum cows was a collaboration with colleagues from Teagasc, Ireland. The authors also thank their colleagues at the Royal Veterinary College for their assistance and encouragement. In particular, Dr Z. Cheng for his help in running the laboratory work, Dr J. Cooke for collecting samples included in the SNP analyses and Drs M. A. Fenwick, S. Lewellyn and W. Chowdhury for immunohistochemistry.

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