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Nutrition and maternal metabolic health in relation to oocyte and embryo quality; critical views on what we learned from the dairy cow model?

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Abstract

Although fragmented and sometimes inconsistent, the proof of a vital link between the importance of the physiological status of the mother and her subsequent reproductive success is building up. High yielding dairy cows are suffering from a substantial decline in fertility outcome during the past decades. For many years, this decrease in reproductive output has correctly been considered multifactorial with factors including farm management, feed ratios, breed and genetics and last but not least, ever rising milk production. Because the problem is complex and requires a multidisciplinary approach, it is hard to formulate straightforward conclusions leading to improvements on the 'work floor'. However, based on remarkable similarities on the pre-implantation reproductive side between cattle and human, there is a growing tendency to consider the dairy cow's negative energy balance and accompanying fat mobilisation as an interesting model to study the impact of maternal metabolic disorders on human fertility and more specifically on oocyte and pre-implantation embryo quality. Taken together the mutual interest of human and animal scientists studying common reproductive problems, the following review serves several aims. First, we will briefly introduce the 'dairy cow case' by describing the state of the art of research into metabolic imbalances and their possible effects on dairy cow reproduction. Secondly, we will try to define relevant *in vitro* models that can clarify certain mechanisms by which aberrant metabolite levels might influence embryonic health. We report on recent advances on embryo metabolism assessment and in the meantime critically elaborate on advantages and major limitations of *in vitro* models used so far. At the end, we open up the discussion on the hurdles to be taken to get these scientific data successfully translated in the field.

Dairy cow fertility on the decline: a well-documented problem

A normal 'nutrition level' and 'metabolic health' of the mother is not only an incentive to ensure female fertility, but also a 'conditio sine qua non' to safeguard successful ovulation, conception, embryo development, maternal receptivity and subsequent maternal-embryonic cross-talk. The observation that maternal metabolic stress or even disorders affect female fertility has been well-documented in high yielding dairy cows (Beam and Butler, 1997; Leroy *et al.*, 2008a). Nutritional requirements increase rapidly with energy priority for the rapidly growing fetus during the last weeks of pregnancy and for the increasing milk production after calving, resulting in a metabolic status of negative energy balance (NEB) post-partum. As extensively described, this is characterized by typical

alterations in plasma metabolite levels of dairy cows (overviewed by Wathes *et al.*, 2007). It is clear that the subfertility problem in high-yielding dairy cows is multifactorial. Santos *et al.* (2010) listed all risk factors associated with a delayed first post-partum oestrus, a reduced conception rate after first insemination and an increased pregnancy loss during the first 60 days of pregnancy. However, more specifically the conflict between metabolic and reproductive needs has been linked with reduced oestrous expression or even anoestrus, ovarian cyst formation and delayed first ovulation (Opsomer *et al.*, 1998; Rhodes *et al.*, 2003). In addition, disappointing conception rates (Bousquet *et al.*, 2004; Santos *et al.*, 2009) and an increasingly high incidence of early embryonic mortality (Bilodeau-Goeseels and Kastelic, 2003; Mann *et al.*, 2006) in these metabolically stressed dairy cows are indicative of problems at the level of the oocyte and/or pre-implantation embryo.

Nutritional and metabolic influences on the central mechanisms governing gonadotropin release are well-studied and reasonably clarified (Schneider, 2004). Disruptions at any level of these connective pathways can disturb the finely tuned endocrine crosstalk in the hypothalamo-pituitary-ovary axis and thereby result in problems with the post-partum onset of oestrus cyclicity, oestrus expression and a timed ovulation (for review: Scaramuzzi *et al.*, 2011).

The effects of metabolic imbalances on pathways regulating oocyte and embryo quality are a rather new field of research. Extensive efforts towards deciphering the link between maternal metabolic health issues and reduced oocyte and embryo quality resulted in highly relevant information, all of which has been extensively reviewed before (Leroy *et al.*, 2012). Altered metabolite concentrations in the mother's blood can be reflected to some extent in bovine and human follicular and reproductive tract fluids (Leroy *et al.*, 2004; Robker *et al.*, 2009; Jungheim *et al.*, 2011; Valckx *et al.*, 2012): the micro-environment in which oocyte maturation, fertilisation and embryo development occurs, respectively. At follicle level, several 'candidate' metabolic factors that might directly influence oocyte development and/or quality have been proposed, such as glucose (Krisher and Bavister, 1998; Sutton-McDowall *et al.*, 2004), non-esterified fatty acids (NEFAs) (Leroy *et al.*, 2005a; Aardema *et al.*, 2011) and urea (De Wit *et al.*, 2001; Leroy *et al.*, 2004). Conditions of heat stress may aggravate the metabolic misbalance in the oocyte's micro-environment (Shehab el Deen *et al.*, 2010). Furthermore, the earliest stages of embryo development are primarily controlled by the quality of the oocyte proper (Watson *et al.*, 1999; Wrenzycki *et al.*, 2007). This implies that metabolic perturbations, induced in (the environment of) the oocyte, may not simply result in suboptimal conception rates, but can also induce changes that become visible at

later stages. In this context, Van Hoeck *et al.* (2011; 2013a) confirmed that elevated NEFA concentrations (typical for conditions associated with upregulated lipolysis, such as NEB, stress or pain) during *in vitro* oocyte maturation not only affect bovine oocyte developmental capacity, but also alter the phenotype of the resulting embryos. More specifically, maturing oocytes for 24h under high saturated NEFA conditions elicits dramatic effects and has led to day 7 embryos with significant altered metabolic footprints both at the level of gene transcription and gene function. These embryos showed a glucose intolerant phenotype while gene transcription for glucose transporters was upregulated. Oxygen consumption dropped significantly. In other words, pre-implantation embryo metabolism can be pre-programmed during the final stage of oocyte maturation. What this implies for the onset of further pregnancy or even for the health of the offspring certainly needs further in depth investigation. The potential importance of elevated NEFA concentrations in the pathway to subfertility both in human and animal settings has been reviewed recently by Van Hoeck *et al.* (2014).

Interestingly, direct effects of metabolite alterations on embryo quality can be expected and recent *in vivo* studies furthermore highlight that the oviduct of dairy cows under metabolic stress is less capable of supporting early embryonic growth stages (Rizos *et al.*, 2010; Maillo *et al.*, 2012). In this context, Leroy *et al.* (2010) showed that dietary induced hyperlipidemic conditions can be harmful for *in vitro* embryo development and metabolism (Leroy *et al.*, 2010) and, more specifically, elevated NEFA concentrations can directly affect the *in vitro* pre-implantation embryo development and gene transcription (reviewed by Van Hoeck *et al.*, 2014).

In addition to micro-environment metabolic perturbation, correct and timed endocrine signalling is crucial in the process of early embryo development. An early onset of the progesterone (P4) rise resulted in significantly better embryo development *in vivo* while extensive *in vitro* research could not find any direct effects of P4 on embryo development (Lonergan, 2011). It is generally known that these P4 levels not only depend on the quality of the corpus luteum, but are also influenced by energy balance, dietary energy intake and liver metabolism. This shows that specific modulations in the oviductal and uterine environment are crucial players linking maternal metabolism to embryo quality. Till day 19 post conception, the bovine embryo will completely rely on the histotrophic composition for all needs (Spencer, 2013) and thus, any change in histotrophic composition might have consequence for the early pre-implantation embryo.

Which knowledge is still lacking and what would be the ideal research model?

Differences between *in vitro* and *in vivo* results … a constant burden …

In terms of the nutritional and metabolic impact on dairy cow fertility, it is clear that new (research) strategies are needed in order to decrease embryonic mortality and pregnancy loss. Therefore, *in vitro* studies are considered as vital tools to gain mechanistic insights. However, is the expertise generated so far, using bovine *in vitro* models, transferable to the much more complex *in vivo* situation? For example, in contrast with the information collected from *in vitro* oocyte maturation studies (Leroy *et al.*, 2005a; Aardema *et al.*, 2011; Van Hoeck *et al.*, 2011), the *in vivo* study of Matoba *et al.* (2012) could not reveal any differences in oocyte quality of cows suffering from lipolytic disorders. Under *in vivo* conditions of maternal metabolic perturbations, both direct and indirect effects of metabolic changes on the oocyte, zygote and embryo can be expected. Elevated plasma NEFA levels for example can jeopardize follicle cell function (Vanholder *et al.*, 2005) and may result in decreased blood P4 concentrations (Yung *et al.*, 1996). The latter can influence the transcriptome signature of the endometrium (Forde *et al.*, 2009; Mesquita *et al.*, 2014). These uterine cells not only determine histotrophic composition, but are also of vital importance for the maternal-embryonic cross-talk and thus maintenance of pregnancy (Mamo *et al.*, 2012).

Also the effect of specific feeding strategies (e.g. additional dietary energy or feed restriction) may depend on the body condition of the mother or on the hormonal conditions around the moment of ovulation (Adamiak *et al.* 2006; Bender *et al.*, 2014). In other words, when designing pharmaceutic (ovarian stimulation or ovulation synchronisation therapies) or nutroceutic strategies to improve embryo survival, all mediators influencing the link between maternal metabolic health status and fertility, should be considered and finely controlled. In order to do so, an integrated approach of *in vitro* and *in vivo* information will be crucial. Finally, it is clear that such strategy will need to be tailor made, based on the specific requirements of each breed and farm.

Short term versus long term *in vitro* oocyte exposure models

Most of the *in vitro* models used so far only investigated the short term effects of elevated NEFA concentrations during final bovine oocyte maturation on oocyte developmental competence and embryo quality (Leroy *et al.*, 2005a, Aardema *et al.*, 2011, Van Hoeck *et al.*, 2011, Van Hoeck *et al.*, 2013a, Van Hoeck *et al.*, 2013b). One may question the physiological relevance of this highly defined model as *in vivo* adverse metabolic conditions may inflict

upon ovarian physiological processes during several days, weeks and even months. Importantly, 22 years ago Britt (1992) hypothesized that follicles developing and growing during the period of NEB early post-partum could be affected by the unfavourable metabolic changes and therefore may contain a developmentally incompetent oocyte. Subsequently, after a growing and maturation phase of several weeks, this inferior oocyte will be ovulated at the moment of the first insemination (Lucy, 2003). This long term, so called "carry over" effect has been substantiated in heat stressed dairy cows, clearly describing that oocyte quality remains poor for several weeks after autumn temperatures and temperature humidity indexes became normal again (Roth, 2008). Only very recently first data were published on long term carry over effects of an episode of NEB in dairy cows. Carvalho *et al.* (2014) reported that cows displaying a higher body condition loss during the first three weeks of lactation had a lower percentage of pregnancies after an ovulation synchronisation strategy (> 1800 observations). Importantly, this effect was not consistent in all farms. More in depth follow up of 70 cows showed that cows significantly losing body weight during the first three weeks of lactation displayed a dramatically lower number of viable and transferable good quality embryos after a superovulation treatment around 100 days post-partum as compared to cows that have moderate or no body weight loss early post-partum. In contrast, there was no difference in the number of ovulations, total embryos collected, or percentage of oocytes that were fertilized. These results are obviously consistent with the hypothesis proposed by Britt but a potential clarifying mechanism is lacking. Further research is needed for example to elucidate the importance of the cumulus cells in the link between maternal metabolism and oocyte quality. Cumulus cells are important for optimal oocyte development and maturation (role in signalling meiotic resumption, energy metabolism, anti-oxidative machinery) and only recently it was shown that these somatic cells may provide the oocyte with entire mRNA molecules (Macaulay *et al.*, 2014). We could recently show that cumulus cell gene expression patterns are predictive for embryo development after tracking the oocytes and zygotes in a complete individual culture system (Bunel *et al.*, 2014).

We hypothesized that these long-term effects of adverse maternal metabolic conditions on oocyte quality could also be explained by a long-term effect of elevated NEFA concentrations during the episode of NEB. By lacking a long-term *in vitro* bovine follicle culture system, we relied on a murine animal model to study the effect of long-term elevated NEFA concentrations, as present in individuals or animals suffering from lipolytic disorders, on the follicle as a whole. This model includes the individual culture of early

secondary pre-antral follicles up until day 13, by which time the follicles reach the antral stage *in vitro*, followed by oocyte isolation, fertilisation and embryo culture. The follicle culture has been validated as a functional follicular unit, much resembling the *in vivo* situation (Cortvrindt and Smitz, 1998, Cortvrindt and Smitz, 2001, Cortvrindt and Smitz, 2002). By using this model, both direct effects at the level of the cumulus-oocyte complex and indirect effects, mediated through an altered granulosa and theca cell function, culminate in a final effect at the level of the oocyte. Importantly, when these follicles are exposed to elevated NEFA concentrations throughout follicle growth or only during the final maturation phase, it was shown that long term exposure (13 days) more severely impairs oocyte developmental competence, compared to short term NEFA exposure (only during the final phase of oocyte maturation (Valckx *et al.*, unpubl. data). This strengthens our impression that not only final oocyte maturation, but also the preceding period of follicular and oocyte growth is pivotal for oocyte developmental competence.

Exposing *in vitro* cultured murine follicles to elevated NEFA concentrations throughout follicle growth from the early secondary until the antral stage, resulted in an altered follicular physiology, only moderately affecting follicular growth and antrum formation. The most pronounced effect was induced by stearic acid (HIGH SA treatment). Elevated NEFA concentrations altered gene expression patterns in Day 13 luteinized granulosa cells, revealing that NEFA exposure mainly affected pathways involved in apoptosis, lipid metabolism, oxidative stress and steroidogenesis, which was also evidenced by P4, oestradiol and inhibin B analyses in spent medium. Most importantly, oocytes originating from NEFA exposed follicles displayed a significantly reduced developmental competence until the blastocyst stage (Valckx *et al.*, 2014a). Furthermore, our most recent study determined the metabolic profile of the embryos that did survive to the morula stage. Embryos, originating from oocytes out of HIGH SA exposed follicles (for 13 days), do not seem to consume any glucose (**Figure 1**) compared to controls (Valckx *et al.*, unpubl. data). Strikingly, the same 'glucose intolerant' feature was previously described in granulosa cells originating from HIGH SA exposed follicles (Valckx *et al.*, 2014a), which suggests again that the follicular environment throughout follicular growth may pre-programme the embryonic metabolic profile. Again, the cumulus cells may play an important role but more research is needed.

As mentioned previously, not only the concentrations but also the type and the ratios of the fatty acids can determine the final effect at the level of the cell. We showed that the

maternal metabolic condition is linked to this fatty acid ratio in the follicular fluid NEFA fraction both in dairy cows (Leroy *et al.*, 2005) as in humans (Valckx *et al.*,2014b).

Although many studies have focussed on the effect of adding amino acids to the culture medium on embryo development (Gardner and Lane, 1996, Lane and Gardner, 1998), much research has also investigated amino acid turnover as a potential marker for embryo quality and developmental potential (Houghton *et al.*, 2002, Sturmey *et al.*, 2008, Sturmey *et al.*, 2010). Amino acid profiling has even been successfully used as a non-invasive marker for DNA damage in porcine and bovine blastocysts, as well as Day 2-3 human embryos (Sturmey *et al.*, 2009). In this regard, amino acid analyses of the spent medium of murine morulae, showed that embryos originating from HIGH NEFA exposed follicles tended to have a higher overall amino acid production (Valckx *et al.*, unpubl. data) which is in agreement with Leese's quiet embryo hypothesis (Leese *et al.*, 2008), stating that embryo quality is best served with a relatively low level of metabolism.

We furthermore investigated the intracellular neutral lipid content (lipid droplets) in murine morulae, by means of a previously validated Nile Red staining technique (Genicot *et al.*, 2005, Leroy *et al.*, 2005b). The results showed no significant differences between treatment groups in the murine long-term follicle exposure model. However, increased lipid accumulation has previously been described in response to elevated NEFA exposure during the final oocyte maturation in bovine morulae (Van Hoeck *et al.*, 2013b) and has been associated with suboptimal mitochondrial function as well as alterations in the relative abundance of developmentally important gene transcripts from stress responsive genes (Abe *et al.*, 2002, Rizos *et al.*, 2003, Leroy *et al.*, 2008b). In bovine blastocysts originating from NEFA exposed oocytes, transcriptome analyses furthermore revealed an upregulation of genes related to lipid synthesis (Van Hoeck *et al.*, 2013b), which might be considered as a first coping mechanisms to shuttle fatty acids away from lipotoxic pathways.

It is clear from the above that the short-term bovine exposure model and the long-term murine follicle exposure model are yielding very complementary results. However mouse and cow oocytes are different (Ménézo and Hérubel, 2002; Bilodeau-Goeseels, 2011) and caution is needed when proposing these models as valuable tools to study human subfertility. Fatty acid beta-oxidation is essential for mouse and bovine oocyte developmental competence and early embryo development (Ferguson and Leese, 2006; Dunning *et al.*, 2010). However, a study comparing the effect of inhibiting beta-oxidation during oocyte maturation between species suggested that fatty acid oxidation is less important for mouse oocyte maturation, compared to bovine and porcine (Paczkowski *et al.*,

2013). Importantly, differences between species add value because identified common or shared pathways are most likely to be of importance for other species.

From the above it should be clear that the micro-environment of the maturing oocyte is crucial in determining its quality and development. However, modelling this in relevant research set-ups remains a challenge. Once the oocyte is fertilized, the developing zygote is also very sensitive to alterations in its environment (oviduct and uterus). However, the latter is probably even more difficult to study.

Oviductal environment, so difficult to study …

The relevance of the oviduct as a reproductive organ has previously been described in detail, being of fundamental importance for a number of important events in reproduction, including: storage (Suarez, 2002), capacitation (Dobrinski *et al.*, 1997; Smith and Nothnick, 1997; Fazeli *et al.*, 2003) and selection of spermatozoa; and harboring final stages of oocyte maturation (Kidson *et al.*, 2003), fertilization and development of the pre-implantation embryo (Ellington, 1991; Hunter, 2003). Even more so, important steps in embryo development, such as DNA demethylation (Beaujean *et al.*, 2004; Fulka *et al.*, 2004; Niemann *et al.*, 2007), embryonic genome activation (reviewed by Graf *et al.*, 2014), and DNA replication are taking place during the first cleavage stages of the early embryo. This makes the pre-implantation embryo sensitive to alterations in its environment (Rizos *et al.*, 2010; Maillo *et al.*, 2012; Matoba *et al.*, 2012), which makes the oviductal conditions highly relevant to consider and possibly even control.

A number of studies have emphasized the importance of the oviductal environmental conditions for the pre-implantation embryo. For example, Rizos *et al.* (2010), Maillo *et al.* (2012) and Matoba *et al.* (2012) indicated that the oviductal conditions within the oviduct of lactating cows may fail to support the early embryo and contribute to early embryonic mortality in such animals. Furthermore, using *in vitro* models, Tse *et al.* (2008) proposed that secretion of embryotrophic factors by oviduct cells contribute to improved blastocyst formation and quality. Edwards *et al.* (1997) proposed that composition of the oviductal environment is adapted according to embryonic requirements. Maillo *et al.* (2012) also investigated oviduct-embryo interactions by means of transcriptomic analysis. The response of whole bovine oviducts to the presence of an embryo was investigated, but no clear changes in gene expression patters could be identified. However, a very specific and local reaction, where the embryo is residing, could not be ruled out.

Leese (1988) described the formation of the oviductal luminal fluid (OLF) as an ultrafiltration from the plasma, enriched with actively secreted products from the outlining epithelial cells

and mixed with a fraction of follicular fluid originating from the ruptured dominant follicle at ovulation. As metabolic conditions can be reflected within the follicular fluid (Leroy *et al.*, 2005a; Leroy *et al.*, 2012), they might also be reflected in the OLF but up until now accurate data are still lacking. *In situ* observation of the oviductal micro-environment has proven to be very difficult and current OLF sampling techniques hold certain limitations. They often require anesthesia, specialized equipment and expertise. Samples are often contaminated with erythrocytes, cellular debris and sampling is often hampered by post-interventional complications (Velazquez *et al.*, 2010) or by post-mortem decay (Leese *et al.*, 2008). On the other hand, most *in vitro* research tools lack representativeness of the complex *in vivo* oviduct. Much more work in that field is urgently needed.

Issues in designing optimal *in vitro* research models also entail the substances investigated. Biochemical factors of interest that are lipophilic form a real challenge to be studied in an aqueous *in vitro* environment.

Studying the effect of lipophilic substances in aqueous conditions, not an easy task

It is clear from the above that for example fatty acids are an interesting player in the link between maternal metabolism (and diet) and fertility. However, it is hard to include lipophilic substances in routinely used, hydrophilic *in vitro* culture media. In the blood, the majority of free fatty acids released at lipolysis bind to albumin, a carrier protein (Richieri and Kleinfelt, 1995; McArthur *et al.*, 1999). Therefore, albumin supplementation to the *in vitro* maturation and culture media is crucial to avoid aggregation and precipitation of the NEFAs (Ulloth *et al.*, 2003). Furthermore, albumin also fulfils a vital role in the uptake and trafficking process of NEFAs at the cellular level. Albumin:NEFA complexes may bind to the cell's albumin receptor and deliver the NEFAs directly to the cell membrane, thereby bypassing the aqueous dissociation of albumin:NEFA to unbound NEFA (McArthur *et al.*, 1999). Not only is the use of albumin 'per se' is important, also the concentration of albumin in the medium determines the effects of NEFA exposure (Trigatti *et al.*, 1995; Sorrentino *et al.*, 1998; Synak *et al.*, 2003). For example supra-physiological albumin:NEFA ratios (e.g. >5) should be avoided because they represent conditions in which NEFAs cannot be released (Cnop *et al.,* 2001).

Furthermore, in a serum-free medium, NEFAs cannot be 'captured' by other lipid fractions, which might result in an artificially increased 'toxic effect'. As such, the extent to which the *in vivo* situation should be mimicked as closely as possible versus the extent to which we try to define etiologic mechanisms and pathways, remains a difficult balance to manage.

When studying the effect of dietary provided fatty acids, similar problems pop up. These fatty acids are carried in lipoproteins and it remains a challenge to mimic this *in vitro*. Furthermore, the lipid fraction to which the fatty acids of interest are bound, may affect the biological response of the target cells. Wonnacott *et al.* (2010) could show that poly-unsaturated fatty acid (PUFA) enriched high density lipoproteins (HDL) added to the culture medium elicited a reduced embryo development and altered embryo gene expression for scavenger receptor class B member 1, low-density lipoprotein receptor and stearoyl-CoA desaturase. However, any sign of net fatty acid uptake was lacking. Later, investigators from the same research group showed that albumin seems to be the sole serum fraction able to support a net fatty acid uptake from the supplemented serum clearly affecting the embryo fatty acid profiles and increasing cellular oxidative stress (Hughes *et al.*, 2011). Also theca cells tend to modify their fatty acid uptake, based on whether the PUFA are delivered by HDL or low density lipoproteins (Hughes *et al.*, 2011).

Researchers have attempted to bypass this problem by adopting an integrated approach of *in vivo* and *in vitro* experimental study-designs. For example, Leroy *et al.* (2010) used a bovine model to investigate whether serum, collected from cows with nutritionally induced hyperlipidaemia, may directly affect embryo quality in an *in vitro* embryo production system. As stated before, Hughes *et al.* (2011) also supplemented PUFA enriched serum to the sheep *in vitro* embryo culture showing that the albumin bound fatty acid fraction is associated with active fatty acid uptake in the cells. In line with this, Valckx *et al.* (2014c) collected human follicular samples from women with different body condition and evaluated the effects of this follicular fluid in a bovine oocyte maturation set-up. Bovine embryo developmental competence data, in response to human follicle fluid exposure, were retrospectively linked to the women's specifics. Follicular fluid from obese women was able to hamper *in vitro* bovine oocyte development and to change gene expression patterns of the resulting bovine blastocyst. Differences in the body mass index of women subjected to ovum pick-up are associated with changes in the fatty acid profile of the follicular fluid. However, these changes were only obvious in the NEFA and the triglyceride fraction (Valckx *et al.*, 2014b, Jungheim *et al.*, 2011).

Maternal metabolism pre-programmes health and performance of the offspring; Relevant in a dairy cow setting?

As mentioned above, the micro-environment of the oocyte affects developmental competence, implantation rate and pregnancy. However, the micro-environment of the very

early developmental stages can also induce pertinent changes in the offspring's metabolism and body functions that can persist into or become evident at later stages: after birth, during puberty or even at adulthood (Latham, 1999; Sinclair and Singh, 2007). As such, maternal metabolic disorders periconception and/or during early pregnancy might cause heritable changes, programming the offspring susceptibility to disease (Chavatte-Palmer *et al.*, 2008). This concept is known as 'Developmental Origins of adult Health and Disease' or DOHaD principle, first described by Barker *et al.* (1989). The consequences of maternal metabolism on subsequent reproductive performance and metabolic health of the offspring have recently been reviewed by Chavatte-Palmer and colleagues (2014).

Not all studies could identify a relationship between the metabolic state of pregnant animals and the offspring's development and risk of metabolic disease. Bossaert *et al.* (2014) investigated the consequences for the offspring due to differences in the maternal metabolic state between non-lactating heifers and lactating cows. While the maternal metabolism could not be related to the conformation and glucose metabolism of the newborn calves under field conditions, one has to keep in mind that the metabolic consequences of malnutrition *in utero* may only emerge around or after puberty.

Adaptive metabolic features, in response to a disturbed metabolic environment, can become permanent through altered patterns of gene expression (Lillycrop and Burdge, 2012). Epigenetic mechanisms, such as DNA methylation, are crucial in the regulation of gene expression and intense epigenetic modifications occur in germ cells and the pre-implantation embryo. These epigenetic marks are also sensitive to inadequate (nutritional) environments. For example, we showed that elevated concentrations of NEFAs, during final oocyte maturation and early embryonic development alter DNA methylation in the resultant blastocysts. Most changes were seen in genes related to metabolism and embryonic development suggesting that adverse environmental conditions can affect further development and perhaps postnatal health and metabolism through epigenetic alterations (Desmet *et al.*, 2014a; 2014b). O'Doherty *et al.* (2014) also observed that genomic imprinting, an epigenetic key event during oocyte/follicle growth, may be affected by the compromised metabolic environment in dairy cows in a state of NEB.

Studying pathways is one thing, suggesting solutions is another? Can we feed for improved oocyte and embryo quality?

A lot of research efforts have been attributed to optimize feeding strategies for the improvement of oocyte and embryo quality. Data have been extensively reviewed before

(Leroy *et al.*, 2008a; Leroy *et al.*, 2008b; Leroy *et al.*, 2012; Dupont *et al.*, 2014; Leroy *et al.*, 2014) and are not repeated here. Wiltbank *et al.* (2014) recently expanded in an interesting review on the importance of energy and protein feeding on oocyte and embryo quality in a dairy cow setting. He presented this paper at the recent meeting of the Brazilian society of embryo transfer (Brazil 2014), the world's hot spot of assisted reproduction in cows. One constant issue in all these review papers is that it remains a challenge to come up with relevant guidelines for farmers. When comparing study results, it is clear that the settings at the farm severely impact on the outcome and the conclusions drawn by the researcher. Sometimes, experimental designs are weak and especially in Europe animal studies are severely underpowered (due to financial constrains). Wiltbank *et al.* (2014) calculated that, to be able to detect a 10% difference in pregnancy rate after first breeding, each treatment group should at least include 180 cows.

Too fat or too lean, too productive or too old (let us call it the "too-behaviour") all leads to specific physiological adaptations. Apparently, reproductive pathways are very sensitive to this "too-behaviour" and many recent studies pointed out how this may directly impact on gamete quality. The dairy cow model proved to be an intriguing model to study the link between a disturbed maternal metabolism and oocyte quality. Intense studies in rodent models and more epidemiological observational studies in human settings all come up with very similar conclusions: being healthy (let us call it "balanced-behaviour") remains the keystone for optimal reproduction. Hippocrates (410 BC) stated, "Fatness and flabbiness are to blame. People of such constitution cannot be prolific …" well before the era of the "omics" arrived.

What is rather new in this research field is the fact that a deviating maternal metabolic health during pregnancy due to a "too-behaviour" not only affects the mother's own reproductive capacities but also the health and the reproductive functioning of the offspring. These altered metabolic or nutritional influences during prenatal development can cause epigenetic changes during the early pre-implantation period, potentially leading to transgenerational effects (Lim and Ferguson-Smith, 2010). Further in utero exposure may even affect the F2 generation (Radford *et al.*, 2014; Heijmans *et al.*, 2008).

As researchers we all realize that changing bad habits in animal production systems (but also in human health settings) are very difficult to accomplish, despite the intriguing and overwhelming amount of scientific data generated. It is clear that the feeding and managing for optimal animal health and comfort usually coincides with very good reproductive outcome. Current strategies are sometimes not scientifically based, too complex and

probably too much driven by economic benefits of certain players in the feed industry and/or having increased milk yield as the major goal.

More subtle strategies may help under specific conditions. We are investigating the effect of strategic feeding of anti-oxidants on oocyte quality under conditions of maternal metabolic stress. Interest has been rising on dietary beta-carotene because of their low content in conserved forages (Chauveau-Duriot *et al.*, 2005), reduced levels in peripartum dairy cows (Akar and Gazioglu, 2006) and promising results on pregnancy rates in dairy cows following daily supplementation (Iwanska and Strusinska, 1997; Arechiga *et al.*, 1998; Gossen and Hoedemaker, 2005; de Ondarza and Engstrom, 2009). Recent data revealed that daily β-carotene supplementation in NEB cows was able to rise β-carotene availability in the micro-environment of the maturing oocyte irrespective of the energy status (De Bie *et al.*, 2014). Previous *in vitro* research revealed a better developmental competence after the addition of anti-oxidants to fatty acid exposed embryos during culture (Rooke *et al.*, 2012).

Conclusions

Embryo metabolism takes centre stage both in animal and human reproductive settings. Excellent research data are produced at an overwhelming speed, showing that the embryo changes it metabolism depending on (or suffering from) mothers metabolic health and diet. Many intriguing research models are out now generating promising data. However, several critical points need to be discussed and controlled to further optimize the relevance and the validity of results generated.

Fantastic research was able to translate the “Barker hypothesis” and the “DOHAD concept” into real (molecular) science, steadily revealing the pathways behind it. Despite the potential epigenetic communication between mother’s metabolism around conception and early pregnancy with health of the offspring, in real life the incidence of the ‘too-behaviour’ is only increasing. Both in animal (farming) and human settings there is still a long, challenging way to go to bring laboratory and real live together.

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Figure caption

Figure 1: Glucose turnover of morula stage embryos originating from oocytes that were exposed to elevated NEFA concentrations throughout follicular growth *in vitro*. Glucose concentrations were determined in culture drops of embryos originating from follicles exposed to BASAL NEFA (28µM stearic acid + 21µM oleic acid + 23µM palmitic acid = 72µM NEFA), HIGH SA (280µM stearic acid) and HIGH NEFA (280µM stearic acid + 210µM oleic acid + 230µM palmitic acid = 720µM NEFA) from the early secondary until the antral stage (12days), after a 24h incubation period and corrected with the glucose concentration in empty culture drops. Positive values indicate glucose production into the medium, whereas negative values indicate glucose consumption from the medium.

Answers to the reviewers' comments

Reviewer: 1

Comments to the Author
This review is generally well written and provides a novel perspective by comparing information on cow, mouse and human. My comments are to do with style/English rather than content.

Line 25. Decades would be used more commonly than decennia
This has been changed to 'decades'.

Lines 28-30. This sentence is a bit convoluted and I was not sure what point they wish to make.
'The complexity of the problem warrants a multidisciplinary approach that, very valuable on its own, often troubles specific conclusions leading to improvements on the 'work floor'.'
has been changed to:
'Because the problem is complex and requires a multidisciplinary approach, it is hard to formulate straightforward conclusions leading to improvements on the 'work floor'.'

Line 51. Remove the word "absolute". Even in late pregnancy other parts of the system are going to need energy such as brain, heart etc.
This has been removed.

Line 94. Another convoluted sentence. Change to " .. (especially the fatty acids) elicit dramatic effects and have led to day 7 embryos…"
This sentence has been changed to: 'More specifically, maturing oocytes for 24h under high saturated NEFA conditions elicits dramatic effects and has led to day 7 embryos with significant altered metabolic footprints both at the level of gene transcription and gene function.'

Line 112 should be significantly
This has been changed.

Lines 184-186. Is this meant to be a separate paragraph?
This is not intended as a separate paragraph, we have changed the format to be included in the intended paragraph.

Lines 209 and 218 use English spelling oestradiol and programme
This has been changed throughout the manuscript.

Line 260. Is this meant to say "even so" as this does not make sense?
This has been changed to 'also very'.

Line 423 should be "the major goal"
This has been changed.

Reviewer 2

A fascinating, though provoking and entertaining read manuscript.

Line 25 - define dicennia; consider just saying decades…
As also outlined by reviewer 1, we changed the wording to 'decades'.

Line 29 - replace troubles with often fails to arrive at
We rephrased this sentence, also based on the comment of reviewer 1 to: 'Because the problem is complex and requires a multidisciplinary approach, it is hard to formulate straightforward conclusions leading to improvements on the 'work floor'.'

Line 111 - consider opening with "In addition to microenvironment metabolic perturbation, correct and timed…."
This has been done.

Line 188 - Add Up "Up until day…"
This was changed to: 'This model includes the individual culture of early secondary pre-antral follicles up until day 13, by which time the follicles reach the antral stage in vitro, followed by oocyte isolation, fertilisation and embryo culture.'

Line 120 replace "young" with "early pre (and post?) implantation"
This has been changed to 'early pre-implantation embryo'. As the embryo relies less on its environment after implantation and more on its direct contact with the mother through the placenta, we did not implement (and post) as suggested.

Line 149 Would be worthy to note breed differences - high yielding HF different to a Jersey or a Nelore…
This has been changed to "specific requirements for each breed and farm." The comment of the reviewer is of course very valuable and interesting. However, discussing the breed differences would deviate the main line of the review too much and it should be avoided to lengthen the manuscript even further. Breed differences in terms of management needs are indeed significant as metabolic footprint and priorities may shift dramatically depending on breed differences. Besides, also the genetic merit for "milk production traits" may have already an effect. It will need a substantial number of extra lines to address this properly.

Line 169 - typo; losing
Has been corrected.

Line 220 - consider rephrasing for sentence style
This sentence was rephrased to: 'As mentioned previously, not only the concentrations but also the type and the ratios of the fatty acids can determine the final effect at the level of the cell.'

Line 236 - morulae is plural of morula
This has been changed throughout the manuscript.

Line 254 - suggest adding that the differences in species adds value since common/shared pathways being identified are the most likely of importance for other species.
The following sentence was added: 'Importantly, differences between species add value because identified common or shared pathways are most likely to be of importance for other species.'

Line 314 - missing IS - Not only IS the…
This has been corrected.

Line 336 - missing HAVE and ADOPTING - Researchers HAVE attempted - - by ADOPTING an
This has been corrected.

Line 342- please add references if these are now accepted.
This article is now accepted and the correct reference was included.

Line 413-416 - suggest adding the recent work from Anne Ferguson-Smith lab to this discussion.
These lines were rephrased and 3 extra references were added:

"What is rather new in this research field is the fact that a deviating maternal metabolic health during pregnancy due to a "too-behaviour" not only affects the mother's own reproductive capacities but also the health and the reproductive functioning of the offspring. These altered metabolic or nutritional influences during prenatal development can cause epigenetic changes during the early pre-implantation period, potentially leading to transgenerational effects (Lim and Ferguson-Smith, 2010; Heijmans et al., 2008). Further in utero exposure may even affect the F2 generation (Radford et al., 2014)"

Heijmans, B.T., Tobi, E.W., Stein, A.D., Putter, H., Blauw, G.J., Susser, E.S., Slagboom, P.E., Lumey, L.H. (2008). Persistent epigenetic differences associated with prenatal exposure to famine in humans. Proceedings of the National Academy of Sciences of the United States of America 105, 17046-17049.
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Radford E.J., Ito M., Shi H., Corish J.A., Yamazawa K., Isganaitis E., Seisenberger S., Hore T. A., Reik W., Erkek S., Peters A. H., Patti M. E., Ferguson-Smith A.C. (2014). In utero effects. In utero undernourishment perturbs the adult sperm methylome and intergenerational metabolism. Science 15;345(6198):1255903

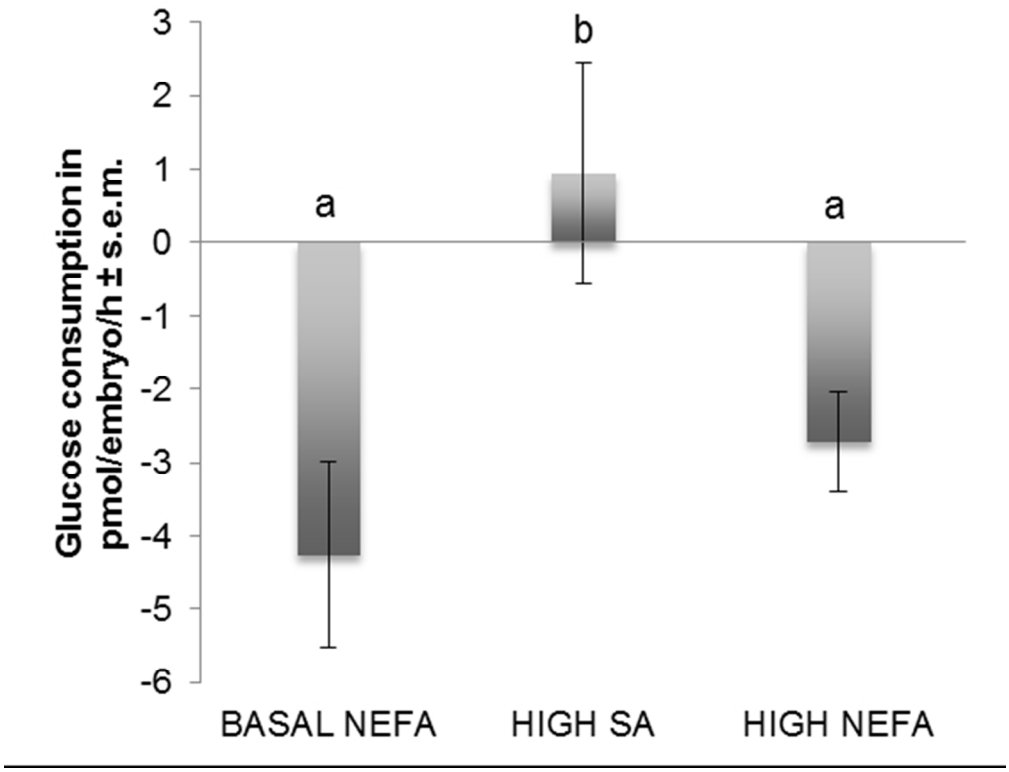

Figure 1: Glucose turnover of morula stage embryos originating from oocytes that were exposed to elevated NEFA concentrations throughout follicular growth in vitro. Glucose concentrations were determined in culture drops of embryos originating from follicles exposed to BASAL NEFA (28μM stearic acid + 21μM oleic acid + 23μM palmitic acid = 72μM NEFA), HIGH SA (280μM stearic acid) and HIGH NEFA (280μM stearic acid + 210μM oleic acid + 230μM palmitic acid = 720μM NEFA) from the early secondary until the antral stage (12days), after a 24h incubation period and corrected with the glucose concentration in empty culture drops. Positive values indicate glucose production into the medium, whereas negative values indicate glucose consumption from the medium.